University of Alberta

Regulation of the SrpABC multidrug and organic solvent efflux pump in *Pseudomonas putida* S12

by

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Abstract

Some organic solvents are extremely toxic to microorganisms and other living cells. They partition into the cytoplasmic membrane and disrupt its structure and function, ultimately resulting in cell death. *Pseudomonas putida* S12 is an unusual soil bacterium that is able to survive high levels of toxic organic solvents. An RND (resistance-nodulation-cell division) type multidrug efflux pump SrpABC plays a key role in the tolerance of *P. putida* S12 to toxic solvents. *srpS* and *srpR* are the two regulatory genes located upstream from the structural genes *srpABC*. Previous evidence suggests that *srpS* encodes the transcriptional repressor protein, while *srpR* encodes the anti-repressor protein. In this study, RT-PCR was used to prove that *srpS* and *R* are polycistronic, as well as to confirm the transcriptional start sites of *srpS* and *srpA*. A mutant strain carrying a newly identified insertion sequence IS*PpuS12* within the repressor gene *srpS* was isolated and characterized. Minimum inhibitory concentration assays were employed to study the substrates of the efflux pump. Finally, a direct protein-protein interaction between SrpS and SrpR was determined by glutathione S-transferase pull-down assays, indicating that the putative anti-repressor SrpR derepresses the SrpABC efflux pump by binding directly to the repressor SrpS.

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List of Abbreviations

α	Alpha
β	Beta
Δ	Delta
λ	Lambda
A	Adenine
Ap	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CAP	Catabolite activator protein
cDNA	Complementary DNA
cfu	Colony-forming unit(s)
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dH_2O	Distilled water
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
G	Guanine
GST	Glutathione S-transferase
h	Hour(s)
His	Histidine
HTH	Helix-turn-helix
IPTG	Isopropyl-β-D- thiogalactopyranoside
IS	Insertion sequence
kb	Kilobase(pair)
kDa	KiloDaltons
Km	Kanamycin
LB	Luria Bertani medium

M	Molar
MCS	Multi-cloning site
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Millilitre
mRNA	Messenger ribonucleic acid
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RND	Resistance-nodulation-cell division
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
T	Thymine
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA buffer
TEMED	Tetramethyl ethylene diamine
Tol	Toluene
µl	Microlitre
v/v	Volume per volume
wt	Wild-type
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1: Introduction

1.0 Introduction

Extremophiles are undoubtedly a group of extraordinary magical organisms. They can adapt themselves to a variety of extreme salinity, pH, temperature, or pressure conditions. In recent years, a new group of extremophiles has been discovered, which can survive in the presence of toxic organic solvents.

Organic solvents exist in the environment. They can either be produced naturally by the process of biosynthesis, or be created by human activities such as industrial and agricultural pollution. Some organic solvents are known to be highly toxic to living cells, including microorganisms, even at a very low concentration. They can accumulate in the cytoplasmic membrane and disorganize it, and also collapse the membrane potential of the cells. For many years people believed that no life could survive in the presence of high amounts of organic solvent. However, this misconception was broken down by the discovery by Inoue and Horikoshi (1989), who found a strain of *Pseudomonas putida* (IH-2000) that could grow actively in the presence of 50% (v/v) toluene.

The mechanisms of organic solvent tolerance in bacteria have been studied by many researchers. These studies are significant for several different reasons. Firstly, they help us to understand how microorganisms survive in extreme environments, and this is important for us to discover the mystery of life. Secondly, one of the major problems in the application of biodegradation to solvent pollution is that high concentrations of organic solvents can have a strong inhibitory effect on the cells; bacteria that can resist

high concentrations of organic solvents have great potential in the treatment of solvent pollution (Collins and Daugulis 1997; Lee et al. 1993). Moreover, organic solvent-tolerant bacteria can also be applied to biocatalysis for the production of certain chemicals in two-phase solvent-water systems (de Bont 1998). For instance, the bioproduction of phenol and cinnamic acid from glucose has been achieved by the application of a solvent-tolerant strain, *P. putida* S12 (Nijkamp et al. 2005; Wierckx et al. 2005). In addition, the study of organic solvent-tolerant bacteria may provide new clues for the design of antimicrobials. Efflux pumps, which play a major role in bacterial solvent tolerance, are also the major mechanism involved in bacterial multidrug resistance. Research with bacteria such as *Pseudomonas aeruginosa* and *P. putida* has shown that some efflux pumps involved in organic solvent tolerance can extrude antibiotics as well (Li and Poole 1999; Ramos et al. 1998; Hearn et al. 2003). Thus the study of organic solvent tolerance mechanisms can also pave the way for the development of antimicrobial therapies.

1.1 Toxic effects of organic solvents

1.1.1 Prediction of toxicity of organic solvents based on $\log P_{ow}$

Organic solvents include low molecular weight aromatic hydrocarbons, cyclic hydrocarbons, aliphatic hydrocarbons and alcohols. The primary site of organic solvent action on cells is the cytoplasmic membrane (Sikkema et al. 1994). Organic solvents can

alter structure and function of the membranes, which leads to the limitation of growth and other cellular activities. For example, tetralin makes the cell membrane much more permeable to ions and inhibits the respiratory enzymes, followed by dissipation of the pH gradient and electrical potential, which finally leads to impairment of cell growth and metabolic functions (Sikkema et al. 1992).

The toxic effects of different organic solvents are closely related to their partitioning characteristics in the membrane. This ability is usually predicted by $\log P_{OW}$, i.e. the logarithm of the partition coefficient of the solvent in a defined octanol-water two-phase system. It has been shown by Sikkema et al. (1994) that there is a parallel relationship between the $\log P_{OW}$ value and the partitioning of solvents in membrane-buffer systems. They chose 10 compounds with $\log P_{OW}$ values ranging from 0 to 4.5, including phenylalanine, benzoic acid, 4-chlorobenzoic acid, 4-toluenesulfonic acid, tetraphenylphosphonium, carvone, toluene, naphthalene, tetralin, and phenanthrene, and the membrane-buffer partition coefficients of these 10 compounds in *E. coli* phospholipid liposomes in potassium phosphate buffer were determined. Afterwards, they compared these membrane-buffer partition coefficients with the octanol/water partition coefficients of these 10 compounds, and found that there was a good linear correlation between the two coefficients. Although some of these 10 compounds are not organic solvents, most organic solvents have $\log P_{OW}$ values within this range. Thus the $\log P_{OW}$ value has been adopted to predict the organic solvent's partitioning ability in the

cell membrane, and hence, the toxicity of the organic solvents.

Usually lipophilic compounds that possess higher affinity for the cell membrane are more toxic than the less lipophilic compounds. For instance, organic solvents that have very low $\log P_{OW}$ values (methanol: -0.79; ethanol: -0.26; acetic acid: -0.25) are only toxic to the cells at high concentrations, whereas organic solvents that have relatively high $\log P_{OW}$ values (hexane: 3.5; toluene: 2.5) are extremely toxic to the cells even at very low concentrations. However, if the compounds' lipophilicity is too high, they are no longer toxic to the cells, because the compounds have to be dissolved in the water phase before they can accumulate in the cell membrane, and if their solubility in water is too low, they are prevented from partitioning into the cells. Hence, the $\log P_{OW}$ values of toxic compounds fall into a certain range. Table 1.1 shows the $\log P_{OW}$ values of a variety of organic solvents. Only the organic solvents with $\log P_{OW}$ values between 1 and 4 are considered extremely toxic to most microorganisms, because on one hand they have relatively high solubility in the aqueous phase, and on the other hand their affinity for the cell membrane is still very strong (Osborne et al. 1990; Vermue et al. 1993).

1.1.2 Organic solvent effects on the cytoplasmic membrane

The accumulation of organic solvents in the cytoplasmic membrane results in a succession of damaging effects to the cells. Firstly, the membrane permeability is increased by organic solvent accumulation, leading to leakage of ions and

Table 1.1. Organic solvents and their $logP_{OW}$ values [a]

Solvent	$logP_{OW}$ value
Methanol	-0.8
Ethanol	-0.3
n-Butanol	0.8
n-Pentanol	1.3
Phenol	1.5
Hexanol	1.9
Chloroform	2.0
Benzene	2.0
Fluorobenzene	2.2
4-Chlorophenol	2.4
Toluene	2.5
Octanol	2.9
Carbon tetrachloride	3.0
Pentane	3.0
Styrene	3.0
p-Xylene	3.0
Ethyl benzene	3.1
Cyclohexane	3.2
Hexane	3.5
Propyl benzene	3.8
Tetralin	3.8
Decanol	4.0
Heptane	4.0
Octane	4.5
Dodecanol	5.0

[a]Adapted from Osborne et al. (1990) and Vermue et al. (1993)

macromolecules from the cells. It was shown by Heipieper et al. (1991) that the cytoplasmic membrane permeability of *E. coli* was increased by the addition of phenol or 4-chlorophenol. These two solvents were observed to cause the loss of potassium ions and ATP from the cells. de Smet et al. (1978) discovered that macromolecules such as phospholipids and several specific cytoplasmic proteins in *E. coli* could be removed by treatment with toluene, and freeze-fracture electron microscopy showed that this effect could be attributed to damage to the cytoplasmic membrane, while the outer membrane still remained intact.

Following the increase of membrane permeability, the cells' membrane potential collapses and the energy status of the cells decreases. Sikkema et al. (1994) showed that the transmembrane pH gradient and electrical potential in artificial membranes were dissipated by cyclic hydrocarbons, leading to the disruption of proton motive force. In addition, Uribe et al. (1990) showed that ATP synthesis in the mitochondria of *Saccharomyces cerevisiae* could be inhibited by cyclohexane.

Moreover, organic solvents can also increase the fluidity of the membrane by partitioning into the membrane and disrupting the interaction between the acyl chains of the membrane fatty acids (Sikkema et al. 1994). Membrane fluidity is closely related to the stability and structure of the membrane, and the functioning of many membrane proteins can be affected by even small changes in membrane fluidity (Zheng et al. 1988). Therefore, the increased membrane fluidity caused by organic solvents can be

detrimental to the cells.

1.2 Strains of organic solvent-tolerant bacteria

Many organic solvent-tolerant bacteria described to date are pseudomonads, including *P. putida* (IH-2000), first described by Inoue and Horikoshi (1989). Since then, several different species of *Pseudomonas* have been reported to survive in organic solvents. Cruden et al. (1992) reported that *P. putida* Idaho is able to grow in the presence of toluene, xylene, 1,2,4-trimethylbenzene, and 3-ethyltoluene when they are provided in a two-phase system at up to 50% (v/v), and it can even use the solvents as sole carbon and energy sources. Other *P. putida* strains that can resist high concentrations of organic solvents, such as *P. putida* S12, *P. putida* DOT-T1E, and *P. putida* GM73, have also been isolated from soil or water environments in recent years (Kim et al. 1998; Ramos et al. 1995; Weber et al. 1993). Solvent-tolerant strains of other *Pseudomonas* species have been discovered as well, including *P. aeruginosa* and *P. mendocina* (Aono et al. 1992; Ikura et al. 1997; Ogino et al. 1994).

Several Gram positive solvent-tolerant genera, such as *Bacillus*, *Rhodococcus*, and *Streptococcus*, have also been isolated recently. Matsumoto et al. (2002) isolated a *Bacillus cereus* strain R1 from soil. This strain can survive in the presence of a second phase of toluene and other organic solvents except aliphatic alcohols, but is unable to use the solvents as carbon and energy sources. Another *Bacillus* sp. strain BC1, isolated from

sea sediment by Sardessai and Bhosle (2003), shows strong tolerance to organic solvents such as chloroform. *Rhodococcus* sp. strain 33, isolated from a contaminated site in Australia, could survive up to 2789 p.p.m. benzene (Paje et al. 1997). This *Rhodococcus* strain, being able to utilize benzene as a sole carbon source and degrade benzene efficiently, shows great potential in the application of bioremediation. Recently, some *Staphylococcus* and *Bacillus* strains have been isolated from soil samples in Alberta (Zahir et al. 2006). These strains are able to survive at concentrations up to 1% (v/v) toluene. Electron microscopy showed that extracellular capsule was produced in *Staphylococcus* sp. strain ZZ1 in the presence of toluene, which could be a mechanism of organic solvent tolerance.

1.3 Organic solvent tolerance mechanisms in bacteria

1.3.1 Organic solvent tolerance mechanisms in Gram positive bacteria

Although Gram positive bacteria have a thick cell wall composed of peptidoglycan, it is not considered to be an effective barrier to organic solvents and other antimicrobial agents, because the size exclusion limit of the cell wall is usually larger than 70 kDa (Scherrer and Gerhardt 1971). However, a number of Gram positive organic solvent-tolerant strains have been isolated in recent years.

The mechanisms of organic solvent tolerance in Gram positive bacteria have not been elucidated very clearly, but active efflux of solvents is considered to be one of the

mechanisms. Three efflux pumps, Bmr, Blt, and Bmr3, have been discovered in *B. subtilis*, and they are involved in the resistance of *B. subtilis* to a variety of antibiotics (Ohki and Murata 1997). But it has not been proven that organic solvents are substrates of these pumps. Matsumoto et al. (2002) have been studying the mechanisms of organic solvent tolerance in *B. subtilis* strain R1. They discovered that the tolerance of strain R1 to toluene is energy-dependent, and inhibitors of energy metabolism such as valinomycin and *o*-vanadate can increase the accumulation level of toluene in the cells. Since valinomycin is a specific inhibitor of the proton motive force, and *o*-vanadate is a specific inhibitor of ATP-binding cassette (ABC)-type efflux pumps, their results suggest that both secondary-type and ABC-type efflux pumps are involved in the tolerance of *B. subtilis* strain R1 to toluene.

Besides efflux pumps, other mechanisms have also been proposed to contribute to the organic solvent tolerance in Gram positive bacteria. Cui et al. (2000) discovered that thickening of the cell wall and alteration of peptidoglycan composition can help a *Staphylococcus aureus* strain to resist vancomycin. Analogous mechanisms may also exist in the tolerance of Gram positive bacteria to organic solvents. However, since the target site of vancomycin is peptidoglycan, while the target site of organic solvents is the cell membrane, whether the alteration of cell wall also contributes to organic solvent tolerance still needs further studies. In addition, it is proposed that solvent metabolism can also play a role in reducing organic solvent toxicity in some Gram positive bacteria

such as *Bacillus* (Sardessai and Bhosle 2002). As mentioned previously, recent evidence from the *Staphylococcus* sp. strain ZZ1 shows that extracellular capsule may be another mechanism of organic solvent tolerance in Gram positive bacteria (Zahir et al. 2006).

1.3.2 Organic solvent tolerance mechanisms in Gram negative bacteria

Many more Gram negative organic solvent-tolerant strains have been isolated than Gram positive strains to date. It is proposed that the potential for solvent tolerance in Gram negative bacteria is higher than in Gram positive bacteria due to the additional outer membrane in Gram negative bacteria (Isken and de Bont 1998). Many studies focused on elucidating the mechanisms of solvent tolerance in Gram negative bacteria. Generally, these mechanisms can be divided into three categories: permeability barrier against organic solvents, solvent degradation and transformation, and active extrusion of solvents by multidrug efflux pumps.

1.3.2.1 Permeability barrier against organic solvents

The initial steps of the damage to the cells caused by organic solvents are the accumulation and penetration of solvents into the cell membrane. As a result, bacteria have evolved the ability to alter their cell membrane to reduce and repair the disruptive effects of solvents.

1.3.2.1.1 Increasing the saturated fatty acid content

In comparison to unsaturated fatty acids, saturated fatty acids have a more ordered steric conformation, which gives the lipid bilayer a denser and more stable structure. Accordingly, increasing the saturated fatty acid content can reduce the fluidity of the membrane and compensate for the destructive effects caused by organic solvents (Isken and de Bont 1998). It has been observed that the degree of saturation of the membrane fatty acids increased in *P. putida* S12 when growing with toluene at a sublethal concentration, but not at a lethal concentration, indicating that the bacteria tend to synthesize saturated fatty acids instead of unsaturated fatty acids to tolerate toluene (Heipieper and de Bont 1994). A similar increase in the relative proportion of saturated fatty acids was observed in *P. putida* strain Idaho, when it was exposed to *o*-xylene (Pinkart et al. 1996). In contrast, the organic solvent-sensitive strain *P. putida* MW1200 showed little increase in saturated fatty acids after the exposure to *o*-xylene. Further studies with solvent tolerant *P. putida* strains showed that the increase in fatty acid saturation became noticeable 15 min after the exposure to *o*-xylene, and the peak proportion was achieved after 2h of exposure to *o*-xylene, indicating that increasing the saturated fatty acid content is a long-term response to solvent exposure (Pinkart and White 1997).

However, it has been observed that the saturation degree of membrane fatty acids decreased in *P. putida* S12 in the presence of nonlethal concentrations of ethanol

(Heipieper and de Bont 1994). This result seems to be contradictory to the organic solvent tolerance mechanisms. Since some of the enzymes required for the biosynthesis of saturated fatty acids in *E. coli* could be inhibited by ethanol (Buttke and Ingram 1980), this unexpected result was proposed to be attributed to the inhibitory effect caused by ethanol rather than the organic solvent-tolerant mechanism of the bacteria (Heipieper and de Bont 1994).

1.3.2.1.2 Isomerizing *cis*-unsaturated fatty acids to *trans*-unsaturated fatty acids

Increasing the saturated fatty acids content is a relatively slow and long-term process for bacteria to resist organic solvents. Before this process starts, another more rapid mechanism is responsible for the tolerance to solvents, i.e. the isomerization of *cis* to *trans* unsaturated fatty acids. As shown in *P. putida* DOT-T1E, the *trans* form fatty acid palmitoleic acid (C16:1, 9) started to be isomerized from the *cis* isomer almost immediately after the cells' exposure to toluene (Ramos et al. 1997).

The conformation of *trans*-unsaturated fatty acids is denser and more stable than *cis*-unsaturated fatty acids, so the *cis* to *trans* isomerization can make the membrane more ordered and decrease the fluidity of the membrane. Heipieper et al. (1992) discovered the *cis* to *trans* unsaturated fatty acid isomerization in *P. putida* P8. They observed that phenol and 4-chlorophenol increased *trans*-unsaturated fatty acid content and decreased *cis*-unsaturated fatty acid content, and that the content of the *trans* isomers

continued to increase even when the concentrations of the solvents were high enough to inhibit the growth of the cells. Incorporation studies with ^{14}C-labelled acetate showed that these nongrowing cells were no longer able to synthesize lipids, indicating that the increase in the content of *trans*-unsaturated fatty acids was the result of direct isomerization from the *cis*-unsaturated fatty acids rather than the result of fatty acid synthesis.

The isomerization of *cis* to *trans* unsaturated fatty acids is catalyzed by *cis-trans* isomerases (Cti). Total DNA Southern blot analysis showed that the *cti* gene, which encodes Cti, is present in several *Pseudomonas* species, including the solvent-tolerant strains *P. putida* P8, *P. putida* DOT-T1E, and *P. mendocina* KR1, and also some solvent-sensitive *Pseudomonas* species (Junker and Ramos 1999). In order to determine whether *cti* is necessary for solvent tolerance, a *cti*-knockout mutant in *P. putida* DOT-T1E was constructed (Junker and Ramos 1999). In contrast to the wild type, this mutant had a much lower survival rate in a shock dose of 0.08% (v/v) toluene. Although the mutant could still grow with toluene in the gas phase, a longer lag phase was observed in comparison with the wild-type strain. The *cti* gene in *P. putida* DOT-T1E was expressed constitutively as shown by primer extension analysis; in-frame fusion of the Cti protein with the periplasmic alkaline phosphatase revealed that this constitutively expressed protein is located in the periplasm. However, the catalytic function of the Cti in *Pseudomonas oleovorans* GPo12 is only active on the crude membranes isolated from

Pseudomonas or *E. coli* in the presence of organic solvents such as toluene, indicating that certain factors, which can be activated by organic solvents, must be involved in the regulation system to enable Cti to perform its catalytic function (Pedrotta and Witholt 1999).

Nevertheless, *cis* to *trans* unsaturated fatty acid isomerization is considered to be a general stress response rather than a specific mechanism for organic solvent tolerance, as it was also found in response to other stress factors such as high temperature, heavy metals, and osmotic stress (Heipieper et al. 1996). Additionally, *cis/trans* isomerization is not considered to be a sufficient mechanism for organic solvent tolerance, because some solvent-sensitive bacteria also have Cti and perform this isomerization when they are exposed to organic solvents (Junker and Ramos 1999; Pinkart et al. 1996).

1.3.2.1.3 Changing the head group composition of phospholipids

Besides the fatty acid composition, alterations in the head group composition of membrane phospholipids can also affect the tolerance of Gram negative bacteria to organic solvents. In *P. putida*, the major classes of phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol or cardiolipin (CL). The transition temperature of CL is higher than PE and PG, thus increasing the percentage of CL is considered to decrease the membrane fluidity and make the membrane more stable. Moreover, CL has a larger headgroup volume than PE,

which can better compensate for the increase in lipid volume caused by organic solvent accumulation (Weber and de Bont 1996). It was shown in *P. putida* S12 that the level of PE and PG decreased, and the level of CL increased significantly in the presence of 1% (v/v) toluene (Weber and de Bont 1996). Similar results were also seen in *P. putida* DOT-T1E when a ^{32}P incorporation experiment was performed (Ramos et al. 1997). In the presence of toluene, more than 90% of the ^{32}P was incorporated in CL, indicating that the head group composition of phospholipids changed by increasing the percentage of CL when the cells were exposed to organic solvents.

1.3.2.1.4 Increasing the basal rate of phospholipid synthesis

Recent studies showed that some organic solvent-tolerant Gram negative bacteria not only can change the content of membrane fatty acid and alter the head group composition to resist organic solvents, but also repair the damaged membrane rapidly by increasing the basal rate of membrane phospholipid synthesis after the exposure to organic solvents. It was shown in *P. putida* Idaho that the synthesis of membrane phospholipids significantly increased when the cells were exposed to *o*-xylene (Pinkart and White 1997). In contrast, the organic solvent-sensitive strain *P. putida* MW1200 only showed a slight increase in phospholipid synthesis after exposure to *o*-xylene, and this was insufficient to maintain the membrane integrity since the total phospholipid content finally decreased by 70%. In the absence of *o*-xylene, both strains showed a similar level

of phospholipid synthesis; but after the exposure to *o*-xylene the synthesis rate increased notably only in the solvent-tolerant strain *P. putida* Idaho, indicating that this increase was induced by *o*-xylene. Thus, increasing the basal rate of phospholipid synthesis is a tolerance mechanism in this solvent-tolerant strain. Another study using $^{13}CH_3$-$^{13}COOH$ incorporation analysis showed that phospholipid synthesis in the toluene-sensitive mutant strain *P. putida* DOT-T1E-109 was diminished significantly in the presence of toluene. In contrast, the phospholipid synthesis rate increased in this mutant strain when growing without toluene, as well as in the solvent-tolerant wild type *P. putida* DOT-T1E when growing with and without toluene (Segura et al. 2004). These results indicate that the reduced solvent tolerance in *P. putida* DOT-T1E-109 is due to the limitation in phospholipid synthesis, and thus confirm that the solvent-tolerant strains can repair the membrane damage by increasing the basal rate of phospholipid synthesis in the presence of organic solvents.

1.3.2.1.5 Outer membrane adaptive mechanisms to organic solvents

In comparison with Gram positive bacteria, Gram negative bacteria have an extra outer membrane, which is mainly composed of phospholipids and lipopolysaccharides (LPS). Although it is not the primary target of organic solvent action on the cells, the outer membrane in Gram-negative bacterial cells is considered to be the first permeability barrier against organic solvents. However, due to the complex structure of

LPS, relatively few studies to date relate to the outer membrane adaptive mechanisms to organic solvents. By measuring the contact angle of water on the cell surface, Weber and de Bont (1996) showed that the cell surface of *P. putida* S12 had a reduced hydrophobicity when growing with acetate, and these acetate-grown cells were much more tolerant towards toluene than the normal glucose-grown cells. These results indicate that acetate can induce a decrease in cell surface hydrophobicity, i.e. outer membrane hydrophobicity, and thus make the cells more tolerant to toluene. Later, it was shown in organic solvent-tolerant mutants of *E. coli* K-12 that the decrease of outer membrane hydrophobicity was due to increased LPS content (Aono and Kobayashi 1997).

In addition to reducing the outer membrane hydrophobicity, decreasing the number of outer membrane-embedded porin proteins is probably another mechanism Gram negative bacteria use to resist organic solvents. Protein F (OprF) is the major outer membrane-embedded porin protein in the organic solvent-sensitive strain *P. aeruginosa* PAO1161. However, it was discovered that OprF was lost in the toluene-tolerant mutant *P. aeruginosa* PAK103. Since it is proposed that toluene may diffuse across the outer membrane through the porin proteins, the loss of these porin proteins probably leads to a stronger tolerance to organic solvents (Li et al. 1995).

More recent studies also showed that the toluene-tolerant strain *P. putida* IH-2000 releases membrane vesicles from its outer membrane to get rid of organic solvents

(Kobayashi et al. 2000). Transmission electron microscopy showed that membrane vesicles with a diameter of about 90nm were produced from the outer membrane of *P. putida* IH-2000 in the presence of toluene, but no membrane vesicles were observed in the absence of toluene. In contrast, no membrane vesicles or small vesicles with a diameter of only 40nm were produced in the toluene-sensitive strains *P. putida* IAM1236^T and *P. putida* strain 32 when growing with toluene. Further analysis showed that the membrane vesicles were composed of phospholipids, LPS, small amounts of membrane proteins, and toluene molecules. Since the surface of the membrane vesicles is covered with LPS, the vesicles are unlikely to be taken up by other cells. Therefore, it was proposed that the shedding of membrane vesicles is used by the bacteria to resist organic solvents.

1.3.2.2 Degradation and transformation of organic solvents

Degradation or transformation of antimicrobial agents is a common mechanism used by many microorganisms in antibiotic resistance. Hydrolases produced by bacteria, such as β-lactamases and macrolide esterases, are used to degrade certain kinds of antibiotics; and group transferases such as acyltransferases and phosphotransferases are used to modify and inactivate antibiotics (Wright 2005). Degradation or transformation of organic solvents has also been observed in some bacteria as a solvent resistance mechanism. For instance, *P. putida* Idaho, which can survive high concentrations of

toluene and xylene, is able to degrade the solvents and use them as carbon and energy sources (Cruden et al. 1992). A mutant strain of *E. coli* which has increased alkylhydroperoxide reductase (AHPR) activity was more tolerant to tetralin and propylbenzene than its parent strain (Ferrante et al. 1995). It was proposed that organic solvents such as tetralin and propylbenzene are not highly toxic to bacterial cells by themselves, but once they enter the cells, they can form hydroperoxides which are highly toxic to the cells. Since hydroperoxides can be transformed into nontoxic compounds by AHPR, it was proposed that the increased activity of AHPR leads to stronger tolerance to these organic solvents in the mutant strain.

However, many of the identified organic solvent-tolerant bacteria cannot degrade or transform the solvents, whereas they are able to survive high concentrations of organic solvents. For example, *P. putida* IH-2000 and *P. putida* S12 cannot degrade or transform toluene at all, but they can still grow actively in the presence of up to 50% (v/v) toluene when it is provided in a two-phase system (Inoue and Horikoshi 1989; Weber et al. 1993). It has been discovered that, although *P. putida* DOT-T1E can utilize toluene as the sole carbon source, metabolizing toluene is unlikely to be involved in toluene tolerance, since a mutant strain unable to metabolize toluene was as tolerant to toluene as the wild-type strain (Mosqueda et al. 1999). Thus, degradation or transformation of organic solvents is not considered to be a major mechanism of solvent tolerance.

1.3.2.3 Active extrusion of organic solvents by efflux pumps

Although a permeability barrier is indispensable for bacteria to resist organic solvents, it is a relatively passive mechanism, and the organic solvents can still diffuse across the barrier after some time, even at a slow rate. Accordingly, there must be some active mechanisms to remove the solvents from the cells. Although solvent metabolism is an active process, its substrate is relatively specific, and this mechanism is not widely utilized by bacteria. Another active process that has been discovered in a number of bacteria is the extrusion of organic solvents by efflux pumps.

The efflux of antimicrobial agents in bacteria as a drug-resistant mechanism was first reported for tetracycline resistance in *E. coli* (McMurry et al. 1980). Later, this mechanism was discovered in many antimicrobial-resistant bacteria. The bacterial drug efflux pumps are divided into five families, including the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the resistance-nodulation-cell division (RND) family. Efflux pumps are located in the cell membrane, and their function is energy-dependent. The ABC family pumps utilize ATP as an energy source, while the pumps in the other families are driven by proton motive force or electrochemical gradient (Kumar and Schweizer 2005).

During the past decade, several efflux systems have been discovered to be involved in bacterial tolerance to organic solvents, including the AcrAB-TolC efflux pump in *E.*

coli (Aono et al. 1998), the MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pumps in *P. aeruginosa* (Li et al. 1998), the SrpABC efflux pump in *P. putida* S12 (Kieboom et al. 1998a), the TtgABC, TtgDEF, and TtgGHI efflux pumps in *P. putida* DOT-T1E (Rojas et al. 2001), etc. These discoveries have helped shed light on the tolerance mechanisms of bacteria to extremely high concentrations of organic solvents.

1.4 RND-type efflux system and its role in organic solvent tolerance

1.4.1 Structure of RND-type efflux pump

All efflux pumps identified to date for organic solvent extrusion belong to the RND family. The RND-type efflux pumps are commonly composed of three parts, a cytoplasmic membrane transporter (CMT) driven by proton motive force, an outer membrane channel protein (OMP) attached to CMT which allows the organic solvent molecules to be extruded outside of the cell, and a periplasmic membrane fusion protein (MFP) which connects CMT with OMP (Fig. 1.1). These three proteins work together, and their assembly and stability depends on the interaction among them. It was found that the formation of MFP-CMT complex is essential for the proper processing of CMT and the stability of OMP in the MexEF-OprN efflux pump of *P. aeruginosa* (Maseda et al. 2002). MFP and CMT have been shown to interact specifically, thus each kind of MFP can only combine with one corresponding kind of CMT. However, OMP is relatively interchangeable among different efflux systems. For instance, OprJ can replace

OprM to make up an efflux pump with MexAB in *P. aeruginosa* (Yoneyama et al. 1998).

It was originally thought that multidrug transporters can only bind their substrates from the inner leaflet of the cell membrane lipid bilayer and then act either as a "cytoplasmic vacuum cleaner" expelling the substrates directly out of the cell, or act as a flippase that flips the substrates into the outer leaflet of the lipid bilayer to diffuse into the extracellular environment (Gottesman and Pastan 1993; Higgins and Gottesman 1992). However, a recent crystal structure determination of the AcrB protein, which is the cytoplasmic membrane transporter of the AcrAB-TolC efflux pump in *E. coli*, strongly suggests that multidrug transporters can probably act as "periplasmic vacuum cleaners" as well. According to Murakami et al. (2002), AcrB contains three open vestibules at the periplasmic surface of the cytoplasmic membrane, connecting the central cavity of AcrB to the periplasm. Furthermore, the vestibules seem to be partially embedded in the outer leaflet of the lipid bilayer. Thus, it was proposed that drugs can also be captured from the periplasm or the outer leaflet of the cytoplasmic membrane, and then be transported into the central cavity of the membrane transporters through the open vestibules. This discovery explains how efflux pumps protect bacterial cells from β–lactam antibiotics, which target the assembly of the peptidoglycan layer in the periplasm. Moreover, toxic hydrophobic compounds like organic solvents are probably removed from the cells through this periplasmic vestibule route as well, since they usually partition into the cell membrane and do not enter into the cytoplasm.

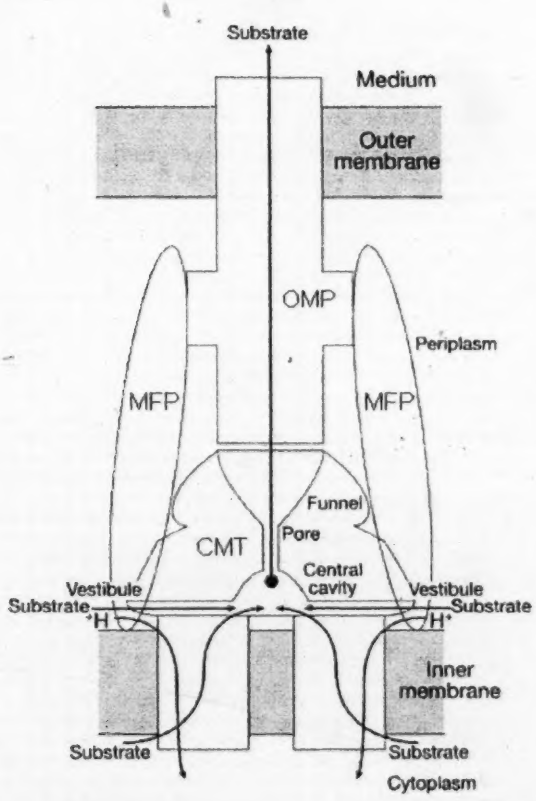


Figure 1.1. Schematic model of the RND-type efflux pump. CM, cytoplasmic membrane; CMT, cytoplasmic membrane transporter; MFP, periplasmic membrane fusion protein; OM, outer membrane; OMP, outer membrane channel protein. Substrates can be captured either from the cytoplasm/inner leaflet of the cell membrane, or from the periplasm/outer leaflet of the cell membrane, and then be transported into the central cavity of CMT before being removed into the extracellular environment through the OMP. Adapted from Murakami et al. (2002).

1.4.2 RND-type efflux systems involved in organic solvent tolerance in Gram negative bacteria

1.4.2.1 AcrAB-TolC efflux system in *E. coli*

The AcrAB-TolC efflux pump plays a major role in multiple antibiotic resistance in some *E. coli* mutants (Okusu et al. 1996). *E. coli* strains are generally not highly tolerant to organic solvents, especially the solvents with a $\log P_{OW}$ value between 1 and 4. However, it was shown that several mutant strains of *E. coli*, which are resistant to low levels of ampicillin, chloramphenicol, nalidixic acid, and tetracycline, have increased tolerance to toxic organic solvents such as cyclohexane and *n*-pentane (Aono et al. 1995). Further research revealed that, as compared with the wild-type strain, these mutants had higher expression levels of AcrA and TolC, which are the MFP and OMP subunits of the AcrAB-TolC efflux pump, respectively. Deletion of the *tolC* gene made the strains hypersensitive to organic solvents, while complementation with *tolC* restored their solvent-tolerant level (Aono et al. 1998). These results indicate that the AcrAB-TolC efflux pump is responsible for the organic solvent tolerance in *E. coli*.

E. coli OST5500, a mutant that lacks *acrB*, is hypersensitive to organic solvents. Several mutant strains constructed from OST5500 showed higher solvent tolerance. It was found that these mutants produced high levels of AcrE and AcrF, which are the MFP and CMT subunits of the AcrEF-TolC efflux pump, respectively. The increased expression from *acrE* and *acrF* was attributed to activation by the insertion sequences

IS*1* and IS*2* that integrated upstream of these two genes. Thus it was proposed that in the absence of *acrAB*, *acrEF* can be activated and make up a pump with TolC to resist organic solvents (Kobayashi et al. 2001). However, since *acrEF* is not active in the wild-type strain, it is still considered that the AcrAB-TolC system plays the major role in solvent tolerance in *E. coli*.

1.4.2.2 Multiple efflux systems in *Pseudomonas* species

1.4.2.2.1 SrpABC efflux system in *P. putida* S12

Active extrusion of organic solvents in bacteria as a solvent tolerance mechanism was first discovered in *P. putida* S12 (Isken and de Bont 1996). Studies showed that the toluene accumulation level was much lower in toluene-adapted *P. putida* S12 cells than in non-adapted cells. However, the toluene accumulation level in adapted cells was remarkably increased by the energy inhibitors potassium cyanide and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), while toluene accumulation in the non-adapted cells was not affected by the energy inhibitors. These results indicated that an energy-dependent solvent efflux system was present in this toluene tolerant strain. The identification of an efflux pump being responsible for organic solvent tolerance in bacteria was also first discovered in *P. putida* S12 (Kieboom et al. 1998a). Research showed that three genes *srpA*, *srpB*, and *srpC* were involved in this efflux system, encoding the MFP, CMT, and OMP subunits of the efflux pump, respectively (Kieboom

et al. 1998a). The SrpABC efflux pump showed extensive homology to other known RND-type multi-drug efflux pumps. The expression of this pump can be induced by a variety of toxic aromatic and aliphatic solvents and alcohols. However, no antibiotic was shown to induce the expression of this efflux pump (Kieboom et al. 1998b). Since the mutant strain JK1 which lacks this pump showed a similar level of resistance to antibiotics as the wild-type strain, it was proposed that the SrpABC efflux pump is not responsible for antibiotic resistance (Isken and de Bont 2000). Later, another RND-type efflux pump ArpABC was identified in *P. putida* S12 (Kieboom and de Bont 2001). This efflux pump contributes to antibiotic resistance in *P. putida* S12 but is not involved in organic solvent tolerance.

1.4.2.2.2 TtgABC, TtgDEF and TtgGHI efflux systems in *P. putida* DOT-T1E

Three different RND-type efflux systems, TtgABC, TtgDEF, and TtgGHI, are involved in the tolerance of *P. putida* DOT-T1E to organic solvents. These efflux pumps are encoded by three operons *ttgABC*, *ttgDEF*, and *ttgGHI*, respectively. The *ttgABC* operon is expressed constitutively at a high level in the absence of organic solvents, and its expression increases slightly in response to organic solvents (Duque et al. 2001). The *ttgDEF* operon is inactive in the absence of organic solvents, and its expression can be induced by aromatic hydrocarbons (Mosqueda and Ramos 2000). The *ttgGHI* operon expresses at a moderate basal level in the absence of organic solvents, and it can be

induced by solvents and reach a high level (Rojas et al. 2001). Both the TtgABC and TtgGHI pumps have a broad range of substrates, including a number of aromatic hydrocarbons and antibiotics such as ampicillin, chloramphenicol, and tetracycline. In contrast, the TtgDEF pump has a relatively narrow range of substrates, as only toluene and styrene have been found to be extruded by this pump. It has been shown that a double mutant lacking both TtgABC and TtgDEF pumps could still survive being shocked with a high dose of toluene, but a single mutant lacking *ttgH* was hypersensitive to toluene. Thus it was proposed that the TtgGHI efflux pump plays a pivotal role in the tolerance of *P. putida* DOT-T1E to organic solvents. The TtgGHI system is highly homologous to the SrpABC system in *P. putida* S12, as a nucleotide sequence comparison of these two systems revealed 98% identity (Rojas et al. 2001).

1.4.2.2.3 MexAB-OprM efflux system in *P. aeruginosa*

The MexAB-OprM system was discovered initially as an antibiotic resistance efflux pump in *P. aeruginosa*. Mutants lacking *mexA* and *oprM* showed an increased susceptibility to tetracycline, norfloxacin, and a number of β-lactam antibiotics, and a mutant overproducing the MexAB-OprM pump was more resistant to these antibiotics (Li et al. 1995). Subsequent studies revealed that this pump is also responsible for the tolerance of *P. aeruginosa* to organic solvents, as the *mexA-mexB-oprM* deletion mutant showed hypersensitivity to solvents such as *n*-hexane and *p*-xylene, and the mutant

overexpressing this pump had increased tolerance to these solvents. Another two efflux systems, MexCD-OprJ and MexEF-OprN, which are not expressed constitutively in *P. aeruginosa*, also contribute to organic solvent tolerance, since overexpression of these two pumps in a mutant lacking MexAB-OprM still provided a low level of tolerance to organic solvents. However, this tolerance level is much lower than that of the wild-type strain, indicating that the MexAB-OprM efflux system is the major contributor to organic solvent tolerance in *P. aeruginosa* (Li et al. 1998).

1.4.2.2.4 EmhABC efflux system in *P. fluorescens*

Recent studies have discovered a new efflux system in *P. fluorescens* cLP6a, which is a polycyclic aromatic hydrocarbon (PAH) degrading strain. This system has been shown to extrude PAHs, including phenanthrene, anthracene, fluoranthene, but not naphthalene (Bugg et al. 2000). PAHs usually have $\log P_{OW}$ values greater than 4 (except the $\log P_{OW}$ value of naphthalene is 3.3), and are not considered highly toxic to microorganisms. However, since *P. fluorescens* cLP6a is a PAH degrader, and takes up PAHs into the cell through passive transport, the efflux system is probably used to regulate the concentration of cell-associated PAHs. Further studies revealed that this system is also an RND-type efflux system, and its three structural genes have been identified and designated *emhABC*. This efflux system has a relatively narrow substrate range, including several PAHs, toluene, and a few hydrophobic antibiotics such as

chloramphenicol, nalidixic acid, rhodamine 6G, dequalinium, and ciprofloxacin (Hearn et al. 2003; Hearn et al. 2006).

1.5 Regulation of the expression of RND-type efflux systems

Since the bacterial efflux systems are energy consuming, their expression has to be tightly controlled under nonselective conditions in order to save energy for the bacteria. In addition, since multidrug efflux systems such as AcrAB-TolC have a broad substrate range, it is suggested that they may even pump out normal metabolites from bacterial cells (Li and Nikaido 2004). Thus it is proposed that sustained overexpression of efflux pumps can be detrimental to cells. In fact, an *E. coli* mutant strain lacking the repressor of the AcrAB-TolC efflux pump was shown to have a slower growth rate than the wild-type strain (Ma et al. 1996). Similarly, it was reported that the efflux pump-overproducing mutants of *P. aeruginosa* and *P. putida* had reduced fitness when compared with their parental strains (Sánchez et al. 2002; Wery et al. 2001). Accordingly, it is important for the bacteria to have their efflux pumps under the control of certain regulatory systems.

1.5.1 Local regulators

The expression of most RND-type efflux pumps is controlled by a local regulator, usually a repressor, whose gene is situated upstream from the efflux pump structural

genes and transcribed divergently from them. The AcrAB-TolC efflux pump in *E. coli* has a repressor gene *acrR* situated upstream from the structural genes *acrAB*. AcrR, the repressor protein encoded by *acrR*, can bind to the *acrR-acrA* intergenic region and repress the expression from the *acrA* as well as the *acrR* promoter, indicating that this repressor is auto-regulated (Ma et al. 1996).

A gene designated *mexR* has been found upstream from the efflux operon *mexAB-oprM* in *P. aeruginosa*. Although this gene shows little similarity to *acrR*, it does show a high homology to *marR*, which encodes the repressor of MarA-dependent multidrug resistance in *E. coli*. The mutant strain lacking *mexR* showed increased expression from the *mexA* promoter in comparison with the wild-type strain, indicating that *mexR* produces a repressor of the *mexAB-oprM* operon. Consistently, this mutant showed stronger antibiotic resistance due to the overexpression of the MexAB-OprM efflux pump (Poole et al. 1996).

Similarly, *ttgR* was identified upstream from the *ttgABC* efflux operon in *P. putida* DOT-T1E. This gene has high homology to *acrR* in *E. coli*, and it is also transcribed divergently from the downstream efflux operon. The TtgR protein encoded by *ttgR* was shown by gene knock-out experiments to be the repressor of the TtgABC efflux pump (Duque et al. 2001). Primer extension tests revealed that the transcription initiation points of *ttgR* and *ttgA* are only separated by 28 base pairs, and their -10 and -35 boxes overlap with each other. DNase I footprinting assay showed that TtgR binds to this

ttgR-ttgA intergenic region, preventing the binding of RNA polymerase, and repressing the transcription of *ttgA* and also *ttgR* itself (Teran et al. 2003). Gel mobility shift assays showed that antibiotics such as chloramphenicol and tetracycline bind to TtgR and release it from its DNA target. Further studies revealed that several structurally unrelated molecules such as butyl paraben, 1,3-dihydroxynaphthalene and plant-derived phloretin and quercetin are also able to release TtgR from the DNA-binding site, indicating that these efflux pump substrates can directly interact with the repressor TtgR and enhance the expression of the TtgABC efflux pump (Teran et al. 2003; Teran et al. 2006).

1.5.2 Global regulators

Many studies have been focusing on the global regulatory system involved in the control of the AcrAB-TolC efflux pump in *E. coli*. As mentioned before, the AcrAB-TolC pump is under the control of the local regulatory gene *acrR*. However, several other genes also contribute to the regulation of this efflux pump. *marA* is a constitutively expressed gene that encodes a regulator controlling over 60 chromosomal genes in *E. coli* (Barbosa and Levy 2000). *marA* and two other genes, *marR* and *marB*, comprise an operon *marRAB*. *marR* encodes a repressor, MarR, which inhibits the expression of the *marRAB* operon, thus it determines the expression level of *marA*, whereas the function of *marB* is still unclear (Alekshun and Levy 1997). Overexpression of *marA* has been shown to activate transcription from the *acrAB* operon and increase the tolerance of *E.*

coli cells to antibiotics (Okusu et al. 1996). In addition, overexpression of two other genes, *soxS* and *robA*, which are homologous with *marA*, also increases the tolerance of *E. coli* cells to organic solvents and antibiotics (Nakajima et al. 1995). Furthermore, a possible *mar-rob-sox* box was found upstream from *tolC*, and overproduction of MarA, SoxS or RobA led to increased expression of TolC (Aono et al. 1998). In conclusion, the AcrAB-TolC efflux pump is positively controlled by a global regulatory system, which consists of *marA*, *soxS* and *robA*.

1.5.3 Two-component / two-regulator systems

A few RND-type efflux pumps have been found to be regulated by two-component systems. A typical bacterial two-component regulatory system is composed of a sensor kinase and a response regulator. The sensor kinase senses environmental alterations and adjusts the phosphorylation state of the response regulator, which is responsible for gene regulation (Nishino and Yamaguchi 2002). The SmeABC efflux pump, which has homology to the MexAB-OprM efflux pump in *P. aeruginosa*, is an RND-type efflux system in *Stenotrophomonas maltophilia*. Upstream from the efflux pump structural genes *smeABC*, two genes have been identified and designated *smeSR*. These two genes are homologous with other identified bacterial two-component regulatory systems. SmeS is the putative sensor kinase, and SmeR is the putative response regulator. SmeR was shown to activate the expression of the SmeABC efflux pump and enhance the multidrug

resistance of this bacterium (Li et al. 2002).

The TtgGHI efflux pump of *P. putida* DOT-T1E also has two regulatory genes *ttgV* and *ttgW*, located upstream from the pump structural genes *ttgGHI* (Fig. 1.2). These two regulatory genes are polycistronic, and they are transcribed divergently from the *ttgGHI* operon (Rojas et al. 2003). Although this arrangement is similar to the SmeABC efflux pump of *S. maltophilia*, the function of *ttgVW* is completely different from that of *smeSR*. A mutant deficient in *ttgV*, but not in *ttgW*, has much higher expression activity from the *ttgGHI* and *ttgVW* promoters, suggesting that *ttgV* encodes a repressor for the expression of *ttgGHI* and itself. A similar result has also been observed in a *ttgVW* double mutant. However, the mutant lacking *ttgW* but with intact *ttgV* did not show any significant changes in the expression level of *ttgGHI* and *ttgVW*. Thus it was proposed that *ttgW* plays no major role in this regulatory system (Rojas et al. 2003). Further studies revealed that TtgV represses the transcription of *ttgGHI* and *ttgVW* by binding to the *ttgV-ttgG* intergenic region and preventing the access of RNA polymerase. Substrates of the TtgGHI pump such as 1-hexanol can release TtgV from its DNA binding site and induce the expression of the efflux pump (Guazzaroni et al. 2004). A recent study (Guazzaroni et al. 2005) showed more compounds that play a similar role as 1-hexanol, including 1-naphthol, 2,3-dihydroxynaphthalene, 4-nitrotoluene, etc. A clear relationship has been observed between the compounds' affinity for TtgV and their efficiency to induce the expression of *ttgGHI*. Effectors that have a high affinity for TtgV, such as 1-naphthol,

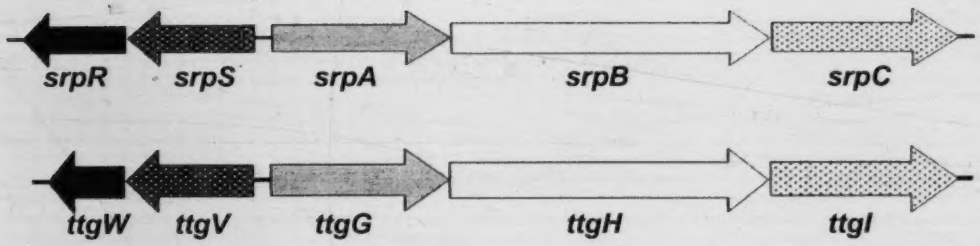


Figure 1.2. Schematic diagrams of the *srpABC-srpSR* cluster and the *ttgGHI-ttgVW* cluster. These two clusters have 98% identity at the nucleotide level. *ttgW* is approximately two-thirds the length of *srpR*, as *ttgW* is probably a pseudogene encoding a truncated protein (Ramos 2004).

were shown to be the most efficient gene inducers (Guazzaroni et al. 2005).

The SrpABC efflux pump in *P. putida* S12, which is highly homologous with TtgGHI in *P. putida* DOT-T1E, was also found to have two regulatory genes, *srpR* and *srpS*, situated upstream and transcribed divergently from the structural genes *srpABC* (Fig. 1.2) (Dennis and Zylstra 1999, GenBank entry AF061937). *srpS* encodes the putative pump repressor SrpS, while the function of *srpR*/SrpR is still undefined. Wery et al. (2001) discovered a 2.6-kb insertion sequence IS*S12* that can insert into the repressor gene *srpS* blocking its expression, and thus enhance the expression of *srpABC*. This result suggests that insertion sequences are probably involved in the regulation of expression of RND-type efflux systems as well.

According to its amino acid sequence, SrpS was predicted to belong to the IclR family of transcriptional regulators. Members of this family contain a helix-turn-helix (HTH) DNA-binding domain of about 60 amino acids in the N-terminal part, as well as an effector binding domain of about 170 amino acids in the C-terminal part. IclR-like proteins can function as transcriptional repressors, activators or proteins with a dual role (Molina-Henares et al. 2006). On the other hand, SrpR was predicted to belong to the TetR family of transcriptional regulators. Members of this family also contain a HTH DNA-binding domain of about 60 amino acids in the N-terminal part, while the C-terminal part is usually involved in binding of inducers or oligomerization. Unlike members of the IclR family, all members of the TetR family whose function is known are

transcriptional repressors (Ramos et al. 2005). The amino sequences of SrpS and SrpR, as well as their featured domains are shown in Fig. 1.3.

1.6 Research objectives

Unlike most RND-type efflux systems in Gram negative bacteria, both the SrpABC and the TtgGHI efflux pumps in *P. putida* have two regulatory genes upstream from the structural genes. Rojas et al. (2003) concluded that *ttgV* encodes a repressor that prevents expression of the *ttgGHI* operon, whereas *ttgW* does not play a significant role in the regulation system. Since the *srpABC-srpSR* gene cluster and the *ttgGHI-ttgVW* gene cluster are highly homologous, it was thought that *srpSR* should perform in the same manner as *ttgVW*. However, Zahir (2005) conducted β-galactosidase assays (Table 1.2) and electrophoretic mobility shift assays, and found that SrpR could apparently derepress the expression of the *srpABC* operon by preventing the binding of SrpS to its DNA target, as well as dissociating the pre-bound SrpS. A model diagram of the proposed transcriptional regulation mechanism of the *srpABC* operon is shown in Fig. 1.4. The two inconsistent hypotheses about the regulation of the TtgGHI and the SrpABC systems could be explained by the later discovery that *ttgW*, but not *srpR*, is probably a pseudogene (Ramos 2004; Ramos et al. 2005). Although *srpR* and *ttgW* share 96% identity at the nucleotide level, *srpR* encodes a 213-amino-acid protein, whereas *ttgW* only encodes a 134-amino-acid protein, and the nucleotide sequence beyond the stop

SrpS (259 aa)

MNQSDENVGKAGGIQVIARAASIMRALGSHPHGLSLAAIAQLVGLPRSTVQRIIN 55

AEELEESVCLALEEEFLVEALGPAGGFRLGPALGQLINQAQSDILSLVKPYLRSL 110

ASLAGDKIYVLDRIVSERELRVVFPIGINVPAAATAAGKVLLAALPDETLQAALG 165

EQLPVFTSNTLRRKALVKQLSEVRQSGFASDLDEHIDGVCSFATLLDTYLGYYSL 220

AVVMPSSRASKQSDLIKKALLQSKQNIERAIGRASKKAP 259

SrpR (213 aa)

MARKTAAEAEETRQRIIDAALEVFVAQGVSDATLDQIARKAGVTRGAVYWHFNGK 55

LEVLQAVLASRQHPLELDFTPDLGIERSWEAVVVAMLDAVHSPQSKQFSEILIYQ 110

GLDESGLIHNRMVQASDRFLQYIHQVLRHAVTQGELPINLDLQTSIGVFKGLITG 165

LLYEGLRSKDQQAQIIKVALGSFWALLREPPRFLLCEEAQIKQVKSFE 213

Figure 1.3. Amino acid sequences of SrpS and SrpR. The predicted effector binding domain of SrpS is indicated by a single line. The predicted HTH DNA-binding motifs of both SrpS and SrpR are indicated by double lines. Sequences were analyzed with the ScanProsite program on the ExPASy proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.org).

Table 1.2. Transcription from the *srpA* promoter determined by β-galactosidase assays [a]

Strain	β-Galactosidase activity (A_{420}/ OD_{600})	
	No toluene	With toluene
S12/*lacZ*	535 ± 8	3014 ± 218
SrpS$^-$/*lacZ*	2700 ± 134	2717 ± 61
SrpR$^-$/*lacZ*	92 ± 4	933 ± 57

[a] Data adapted from Zahir (2005). SrpS$^-$/*lacZ*, SrpR$^-$/*lacZ* and S12/*lacZ* are three *srpA-lacZ* reporter gene fusion strains. β-Galactosidase assays were performed with these three strains to determine the expression level of the *srpA* promoter in the presence and absence of toluene in the wild-type strain as well as the *srpS* deletion and *srpR* deletion mutants. The results suggest that SrpS represses while SrpR derepresses the transcription of the *srpA* promoter. Although these results also suggest that toluene directly interacts with SrpS to increase the expression of the *srpABC* operon, the transcriptional level does not return to the wild-type level in the SrpR$^-$ mutant even when toluene is added, suggesting that both toluene and an intact SrpR are needed for complete derepression of the *srpABC* operon.

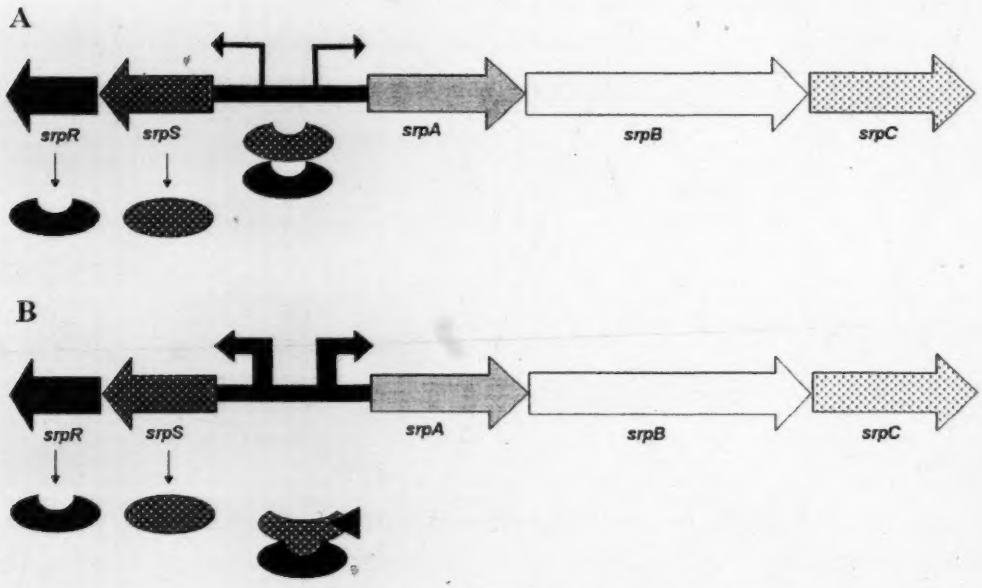


Figure 1.4. Proposed mechanism of transcriptional regulation of the *srpABC* operon in *P. putida* S12. Figure adapted from Zahir (2005). (A) In the absence of toluene, SrpR binds to SrpS and changes the conformation of SrpS. This reduces the binding ability of SrpS to its DNA target and causes a slight increase in dissociation of SrpS pre-bound to the DNA, allowing some derepression of the *srpS* and *srpA* promoters. (B) In the presence of toluene, toluene binds to SrpS in the SrpS-SrpR complex and alters the conformation of SrpS to inhibit the binding of SrpS to its DNA target. This strongly increases the transcription level from the *srpS* and *srpA* promoters. The angled black arrows represent the promoters of *srpS* and *srpA*, with the thickness of the arrows indicating the level of transcription. The black triangle represents toluene.

codon of *ttgW* remains 96% identical to the sequence of *srpR*. Therefore, *ttgW* encodes a truncated protein, while *srpR* probably encodes the intact regulator.

The objective of this research was to further study the two regulatory genes *srpSR* in *P. putida* S12, and to elucidate the mechanism of this newly-identified two-regulator system. RT-PCR was employed to determine if *srpR* and *S* are polycistronic, as well as to confirm the transcriptional start sites of *srpS* and *srpA*. PCR and Southern hybridization analysis were used to detect insertion sequences (IS) within the *srpRS* region in cells surviving toluene shock. Survival frequency tests were performed to compare the survival rate between the *srpS*::IS mutant and the wild-type strain in response to toluene shocks. Minimum inhibitory concentration (MIC) assays helped to determine the substrates for the SrpABC efflux pump. Finally, to study if the putative anti-repressor SrpR directly interacts with the repressor SrpS, an in vivo bacterial two-hybrid system and an in vitro glutathione S-transferase pull-down assay were used to detect the protein-protein interaction between SrpR and SrpS.

Chapter 2: Materials and Methods

2.1 Bacterial strains, plasmids, and culture conditions

2.1.1 Bacterial strains

The *Escherichia coli* and *Pseudomonas putida* strains used in this study are listed in Table 2.1.

2.1.2 Plasmids

Cloning vectors and recombinant plasmids used in this study are listed in Table 2.2, along with their relevant characteristics.

2.1.3 Culture conditions

Bacterial strains were routinely grown in Luria-Bertani (LB) medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl) (Sambrook et al. 1989). Solid medium contained 1.5% (w/v) agar. Ampicillin (100 μg/ml) or kanamycin (50 μg/ml for *E.coli*, and 250 μg/ml for *P. putida*) was added to maintain different plasmids. 2x YT medium (1.6% [w/v] tryptone, 1% [w/v] yeast extract, 0.5% [w/v] NaCl) was used for protein expression in *E. coli* strains. *E. coli* cultures were incubated at 37°C, while *P. putida* cultures were incubated at 30°C. Liquid cultures were shaken on a horizontal shaker at 200 rpm. When *P. putida* culture plates were supplied with toluene via the gas phase, a sealed glass chamber was used, and saturated toluene vapour was achieved by

Table 2.1. Bacterial strains used in this study

Strain	Relevant characteristics	Reference / source
E. coli DH5α	F^- φ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen
E. coli BL21	F^-, *ompT*, *hsdS* (r_B^-, m_B^-), *gal*	Amersham
E. coli M15	Nal^S, Sm^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+	Qiagen
E. coli DHM1	F^- *cya-854 recA1 endA1 gyrA96 (*Nal^R*) thi1 hsdR17 spoT1 rfbD1 glnV44(AS)*	(Karimova et al. 1998)
E. coli BTH101	F^- *cya-99 araD139 galE15 galK16 rpsL1 (*Str^R*) hsdR2 mcrA1 mcrB1*	(Karimova et al. 1998)
P. putida S12	Wild-type, *srpABC*$^+$, *srpR*$^+$, *srpS*$^+$	(Hartmans et al. 1990)
P. putida JK1	*srpB*::Tn*Mod*-KmO	(Kieboom et al. 1998a)
P. putida S12TS	*srpS*::IS*PpuS12*	This study
P. putida S12DM	*srpS*::IS*PpuS12* *srpB*::Tn*Mod*-KmO	This study

Table 2.2. Cloning vectors and plasmids used in this study

Plasmid	Relevant characteristics	Reference / source
pCR2.1-TOPO	TOPO TA cloning vector for direct insertion of PCR products, blue/white screening, Ap^R, Km^R	Invitrogen
pJD101	Derived from a *Bam*HI digestion of *P. putida* JK1 chromosome. Contains *srpR* through partial open reading frame of Tn*Mod*-KmO plasposon-mutated *srpB*.	(Kieboom et al. 1998a)
pJD102	Derived from a *Pst*I digestion of *P. putida* JK1 chromosome. Contains partial open reading frame of Tn*Mod*-KmO plasposon-mutated *srpB* through *srpC*.	(Kieboom et al. 1998a)
pJD203	Constructed by digestion (*Eco*RV + *Bam*HI) and ligation of pJD101 and pJD102. Contains partial open reading frame of Tn*Mod*-KmO plasposon-mutated *srpB* through *srpC*.	This study
pBBR1MCS-2	Broad host-range cloning vector, Km^R	(Kovach et al. 1995)
pBBR1-2-*srpR*	The entire coding region of *srpR* cloned into pBBR1MCS-2 (*Eco*RI - *Bam*HI).	This study

pBBR1-2-*srpS*	The entire coding region of *srpS* cloned into pBBR1MCS-2 (*Eco*RI - *Bam*HI).	This study
pBBR1-2-*srpRS*	The entire coding region of *srpR* and *srpS* cloned into pBBR1MCS-2 (*Eco*RI - *Bam*HI).	This study
pKT25	Km^R. Cloning vector for the bacterial two-hybrid system. Encodes the T25 fragment of *B. pertussis* adenylate cyclase. Contains an MCS downstream from T25 to allow fusions at the C-terminal of the T25 polypeptide.	(Karimova et al. 1998)
pUT18	Ap^R. Cloning vector for the bacterial two-hybrid system. Encodes the T18 fragment of *B. pertussis* adenylate cyclase. Contains an MCS upstream from T18 to allow fusions at the N-terminal of the T18 polypeptide.	(Karimova et al. 1998)
pUT18C	Ap^R. Cloning vector for the bacterial two-hybrid system. Encodes the T18 fragment of *B. pertussis* adenylate cyclase. Contains an MCS downstream from T18 to allow fusions at the C-terminal of the T18 polypeptide.	(Karimova et al. 1998)
pKT25-*srpS*	The entire coding region of *srpS* cloned into pKT25 (*Bam*HI - *Kpn*I).	This study

pUT18-*srpR*	The entire coding region of *srpR* cloned into pUT18 (*Kpn*I - *Kpn*I).	This study
pUT18C-*srpR*	The entire coding region of *srpR* cloned into pUT18C (*Bam*HI - *Kpn*I).	This study
pKT25-*srpR*	The entire coding region of *srpR* cloned into pKT25 (*Bam*HI - *Kpn*I).	This study
pUT18-*srpS*	The entire coding region of *srpS* cloned into pUT18 (*Bam*HI - *Kpn*I).	This study
pUT18C-*srpS*	The entire coding region of *srpS* cloned into pUT18C (*Bam*HI – *Kpn*I).	This study
pKT25-zip	The leucine zipper domain sequence of the yeast transcription factor GCN4 cloned into pKT25.	(Karimova et al. 1998)
pUT18C-zip	The leucine zipper domain sequence of the yeast transcription factor GCN4 cloned into pUT18C. The plasmids pKT25-zip and pUT18C-zip serve as positive controls for the bacterial two-hybrid system.	(Karimova et al. 1998)
pGEX-4T-1	Ap^R. Cloning vector for overexpression of N-terminal GST-fusion proteins.	Amersham
pGEX-*srpR*	The entire coding region of *srpR* cloned into pGEX-4T-1 (*Bam*HI - *Eco*RI).	This study
pQE31	Ap^R. Cloning vector for overexpression of N-terminal 6xHis-fusion proteins.	Qiagen

pQE31-*srpS*	The entire coding region of *srpS* cloned into pQE31 (*Bam*HI - *Kpn*I).	This study
pQE31-*arpR*	The entire coding region of *arpR* cloned into pQE31 (*Sst*I - *Pst*I).	This study
pREP4	Km^R. Introduced into *E. coli* M15. Constitutively expresses LacI.	Qiagen

adding toluene to the bottom of the chamber. When *P. putida* liquid cultures were supplied with certain concentrations of toluene, the culture flasks were sealed with foil covered stoppers.

2.1.4 Preparation of frozen bacterial stocks

All bacterial strains were grown overnight on LB agar with appropriate antibiotics, and then suspended in 1 ml LB broth with 20% (v/v) glycerol. The cells were then transferred to cryogenic vials and stored at -80°C.

2.2 DNA isolation and transformation

2.2.1 Isolation of total genomic DNA from *P. putida*

Total genomic DNA was isolated from *P. putida* strains by the hexadecyl trimethyl ammonium bromide (CTAB) procedure (Ausubel et al. 1991). Cells grown on agar plates or in liquid broth were harvested and suspended in 567 µl TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Subsequently, 30 µl of 10% (w/v) SDS and 3 µl of 20 mg/ml proteinase K were added to the cell suspension, mixed by pipetting gently and incubated at 37°C for 1 h. The cell suspension was then mixed with 100 µl 5 M NaCl and 80 µl of CTAB solution (10% CTAB, 0.7 M NaCl) and incubated at 55°C for 10 min to allow the selective precipitation of proteins, polysaccharides, and cell wall debris. Following the

incubation, 800 µl 24:1 chloroform:isoamyl alcohol was added, mixed gently and centrifuged for 5 min at 13200 rpm. The top aqueous phase was subsequently extracted with 400 µl phenol and 400 µl 24:1 chloroform:isoamyl alcohol. The aqueous phase was then transferred to a fresh tube and mixed gently with 450 µl isopropanol to precipitate the DNA, which was then collected by centrifugation at 13200 rpm for 5 min. The DNA pellet obtained was washed with 500 µl 75% ethanol, vacuum dried for 15 min and resuspended in 100 µl sterile milli-Q H_2O.

2.2.2 Plasmid DNA preparation from *E. coli*

Plasmid DNA was isolated from *E. coli* cells using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Mississauga, ON) according to the manufacturer's instructions.

2.2.3 Transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* DH5α cells (Invitrogen Corp., Carlsbad, CA) were transformed according to the manufacturer's instructions. The heat shock condition was 42°C for 45 seconds. When required for blue-white screening, cells were spread on LB plates with 100 µl of 100 mM IPTG and 10 µl of 0.1 mg/ml X-gal.

2.2.4 Electroporation of *E. coli* and *P. putida* strains

For electroporation, a single colony was used to inoculate 5 ml LB broth and the

culture was grown overnight at 30°C. On the following day, the culture was diluted 1:50 and grown until the OD_{600} reached 0.6. Cells were harvested from a 1 ml sample by centrifugation at 5000 rpm for 4 min. The supernatant was discarded and the cells were washed with 0.5 ml 10% (v/v) glycerol and centrifuged as before. The wash step was repeated two additional times and the cells were resuspended in 80 µl 10% (v/v) glycerol before plasmid DNA (2-10 µl) was added and mixed gently. The mixture was then transferred to a 0.1 cm Gene Pulser Cuvette (Bio-Rad Laboratories, Hercules, CA) and electroporated using a Bio-Rad MicroPulser. Immediately afterwards, 0.5 ml SOC medium (Sambrook et al. 1989) was added to the cuvette and mixed with the cell suspension. Subsequently, the mixture was transferred to a 1.5 ml microfuge tube prior to incubation on a shaker (200 rpm) at 30°C (*P. putida*) or 37°C (*E. coli*) for 1 h. Following the incubation, 5-200 µl aliquots were spread on LB plates containing the appropriate antibiotic. The plates were then incubated overnight and checked for colony formation.

2.3 DNA analysis

2.3.1 Polymerase chain reaction (PCR)

PCR was used to amplify the DNA fragments for cloning and sequencing reactions. In addition, PCR was also used to check for the presence of insertion sequences or transposons, as well as the correct inserts in recombinant plasmids and integration of the

Table 2.3. Oligonucleotide primers used in this study

Primer	Sequence 5'-3' [a]	Use
#1 SrpS1	TTGGAGGTGAATACTGG	Used with primer #2 to PCR amplify a 203 bp fragment within *srpS*, which includes the insertion site of IS*S12*. Also used in RT-PCR to amplify the first-strand cDNA of *srpS*.
#2 SrpS2	TCGGTCTGCCTGGCTTCT	Used together with primer #1, #5, or #16 in PCR.
#3 SrpS right	ACCACTCTGCCTCACTTCG	Used with primer #4 to PCR amplify a 537 bp fragment within *srpS*, which includes the #1 insertion site of IS*PpuS12*. Also used in RT-PCR to amplify the first-strand cDNA of *srpS*.
#4 SrpS left	ATCCAGGTCATCGCCAG	Used together with primer #3 in PCR.
#5 SrpS3	CGATAGCACGCTCTATATTCTG	Used with primer #2 to PCR amplify a 436 bp fragment within *srpS*, which includes the #1 and #2 insertion site of IS*PpuS12*.
#6 SrpS IS right	TGCCGTCCTTGTTCTTG	PCR amplifies a 352 bp fragment within IS*PpuS12* to be used as the probe for Southern blotting analysis.
#7 SrpS IS left	ATCGAGCCGCACTACCC	

#8 SrpR-001	GAGCACTGGCAATCTAAC	PCR amplifies a 1625 bp fragment encompassing the entire coding sequence of *srpR* and *srpS*.
#9 SrpS-002	AGAGTCTACCATTATGCGAT	
#10 SrpB1	CCAAAATACCCAGAGGAACA	PCR amplifies a 392 bp fragment within *srpB*, which includes the insertion site of the plasposon Tn*Mod*-KmO.
#11 SrpB2	AATCTCCCCTGAGGACTTCG	
#12 BBR-R-5 Eco	GGGAATTCTATGGCCAGAAAGAC	PCR amplifies a 659 bp fragment encompassing the entire coding sequence of *srpR* to clone into pBBR1MCS-2.
#13 BBR-R-3 Bam	CCGGATCCTCACTCGAAG	
#14 BBR-S-5 Eco	CCGAATTCTATGAACCAATCAG	PCR amplifies a 800 bp fragment encompassing the entire coding sequence of *srpR* to clone into pBBR1MCS-2.
#15 BBR-S-3 Bam	TTGGATCCTCTAGGGAGCTTTC	
#14 BBR-S-5 Eco	CCGAATTCTATGAACCAATCAG	PCR amplifies a 1446 bp fragment encompassing the entire coding sequence of *srpR* and *srpS* to clone into pBBR1MCS-2.
#13 BBR-R-3 Bam	CCGGATCCTCACTCGAAG	
#16 R0	CGCCGATTGAGGTTTGAAG	Used in RT-PCR to amplify the first-strand cDNA of *srpR* or *srpRS*.
#17 R2	AGGCGGAGGAGACAAGA	Used with primer #16 to PCR amplify the cDNA of *srpR*.
#18 SF1	CCGTTGGTCGAGGTTTACC	Used with primer #3 to determine the transcription start site of *srpS*.
#19 SF2	CCAGAGCAGCCTCGATCA	Used with primer #3 to determine the transcription start site of *srpS*.

#20 SF0	TGCTGAATCGTAATGCGGT	Used with primer #3 to determine the transcription start site of *srpS*.
#21 AR	CGTGGGTCAATCTGATAAAG	Used in RT-PCR to amplify the first-strand cDNA of *srpA*.
#22 AF1	AGACTCTACCGCATTACGAT	Used with primer #21 to determine the transcription start site of *srpA*.
#23 AF2	ATTACGATTCAGCAATAGCC	Used with primer #21 to determine the transcription start site of *srpA*.
#24 AF0	ATCGCATAATGGTAGACTCT	Used with primer #21 to determine the transcription start site of *srpA*.
#25 SrpR 5Kpn	TTGGTACCGATGGCCAGAAA	PCR amplifies a 652 bp fragment encompassing the entire coding sequence of *srpR* to clone into pUT18.
#26 SrpR 3Kpn	CTCACGGTACCGATTTGACTTG	
#27 SrpR 5Bam	TTGGATCCTATGGCCAGAAAGAC	Used with primer #26 to PCR amplify a 652 bp fragment encompassing the entire coding sequence of *srpR* to clone into pKT25 or pUT18C.
#28 SrpS 5Bam	AAGGATCCTATGAACCAATCA	PCR amplifies a 794 bp fragment encompassing the entire coding sequence of *srpS* to clone into pKT25, pUT18, pUT18C, or pQE31.
#29 SrpS 3Kpn	CTTATCTAGGGTACCTTCTTCGAC	
#30 AR5Sst	AAGAGCTCGATGGTCCGTC	PCR amplifies a 657 bp fragment encompassing the entire coding sequence of *arpR* to clone into pQE31.
#31 AR3Pst	GGCTGCAGCAAAGTGTCAT	

#32 R5Bam	AAGGATCCATGGCCAGAAAGAC	PCR amplifies a 649 bp fragment encompassing the entire coding
#33 R3Eco	GGGAATTCGGATTTGACTTGC	sequence of *srpR* to clone into pGEX-4T-1.

[a] Engineered restriction sites are underlined.

plasmid DNA into the bacterial chromosomes. Oligonucleotide primers and their uses in this study are listed in Table 2.3. Reactions were performed in 50 µl or 100 µl volumes in 0.2 ml tubes using the Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). A standard PCR reaction contained 1 x High Fidelity PCR Buffer (Invitrogen), 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.5 µM of each primer, 1-2 U of Platinum *Taq* DNA polymerase High Fidelity (Invitrogen), and sterile milli-Q H_2O to adjust the volume. For template DNA, 2-4µl of a 1/50 dilution of chromosomal DNA or 1-2µl of a 1/50 dilution of plasmid DNA was used. A general PCR program was as follows: a 2 min denaturation period at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 min per kb of DNA template at 68°C; a final 2 min extension period at 68°C at the end of the program.

2.3.2 Purification of the PCR amplified DNA fragments

DNA fragments amplified for use in sequencing reactions, restriction endonuclease digestions, and TOPO cloning reactions were purified with the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions.

2.3.3 Agarose gel electrophoresis of DNA

DNA samples were analyzed on 0.7% (w/v) agarose, 1x TAE (40 mM Tris-acetate, 1 mM EDTA) gels. Prior to loading, samples were mixed with 1x loading buffer (0.25% bromophenol blue and 40% sucrose).Gel electrophoresis was conducted in 1x TAE at a

voltage of 100 V - 120 V. The 1 Kb Plus DNA Ladder (Invitrogen) or the GeneRuler 1 kb DNA Ladder (Fermentas Inc., Burlington, ON) was used as the molecular weight marker. Following electrophoresis, gels were stained with ethidium bromide for visualization using a UV transilluminator.

2.3.4 DNA purification from agarose gels

DNA fragments were purified from agarose gels when necessary, using the GeneClean II kit (Q-BIOgene, Carlsbad, CA) according to the manufacturer's instructions.

2.3.5 Cloning of DNA

Restriction endonuclease digestions of plasmid DNA and PCR products were performed with enzymes from Invitrogen according to the manufacturer's instructions. For ligation reactions, the mixtures were prepared in volumes of 10-20 µl and consisted of a 3:1 ratio of the insert to vector DNA, 1x ligation buffer (300 mM Tris-HCl [pH 7.8], 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP) and 1 U of T4 DNA ligase according to the manufacturer's recommendations (Promega, Madison, WI). Reactions were carried out at 16°C for 4-8 h before the mixtures were transformed into *E. coli* or *P. putida* cells. In some cases, PCR products amplified with *Taq* polymerase were directly cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions.

2.3.6 DNA sequence analysis

Automated DNA sequencing was performed with the DYEnamic ET kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Reaction mixtures were prepared in 20 µl volumes in 0.2 ml tubes, containing 4 µl template DNA (plasmid or purified PCR product), 1 µl of 50 µM primer, 11 µl sequencing buffer (20 mM Tris [pH 9.0], 5 mM $MgCl_2$. $6H_2O$), and 4 µl ET mix. Sequencing reactions were performed using the Mastercycler gradient thermocycler (Eppendorf) with the reaction program consisting of 25 cycles of 20 seconds denaturation at 95°C, 15 seconds annealing at 50°C and 1 min elongation at 60°C. Reactions were stopped by the addition of 2 µl NaOAc/EDTA, and the samples were transferred to 1.5 ml tubes and precipitated with 80 µl of 95% ethanol at -20°C for 15 min before harvested by centrifugation. The pellets obtained were vacuum dried for 15 min and provided to the Molecular Biology Service Unit (MBSU), University of Alberta, for automated sequencing using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.3.7 Radioactive labeling of DNA

The linear double stranded (ds) DNA used as a probe for Southern hybridization analysis was obtained by PCR amplification with primers #6 & #7 from *P. putida* S12TS chromosomal DNA, and labeled by the random primer labeling method using the Hexanucleotide Mix kit (Roche Deagnostics GmbH, Penzberg, Germany) according to

the manufacturer's instructions. The reaction mixture consisted of 25 ng denatured target DNA, 60 µM of each of dATP, dGTP, and dTTP, 50 µCi [α-^{32}P]-dCTP (Amersham), 1x Hexanucleotide Mix, 1 µl Klenow enzyme, and sterile milli-Q H_2O up to 20 µl final volume. The reaction was incubated at 37°C overnight, and stopped by heating to 65°C for 10 min. Unincorporated nucleotides were removed using the ProbeQuant G-50 micro columns (Amersham).

2.3.8 Southern hybridization analysis

Total genomic DNA from *P. putida* S12TS or *P. putida* S12 was digested by restriction endonucleases and separated on an agarose gel. Following electrophoresis, the DNA was transferred onto a Hybond-N nylon membrane (Amersham) using the Vacuum Transfer System VT-10 (Tyler Research Instruments, Edmonton, AB) in 20x SSC transfer solution (3 M NaCl, 0.3 M sodium citrate) for 30 min. Subsequently, the membrane was prehybridized in a glass hybridization bottle (Fisher Scientific International Inc., Hampton, NH) with hybridization solution (1 mM EDTA, 0.5 M Na_2HPO_4, 7% [w/v] SDS) at 65°C for 5 min. For hybridization, radioactively labeled DNA probe was added to fresh hybridization solution, and the membrane was incubated at 65°C overnight. After that, the membrane was washed twice for 45 min per time with wash buffer I (1 mM EDTA, 40 mM Na_2HPO_4, 5% SDS) and twice for the same period with wash buffer II (1 mM EDTA, 40 mM Na_2HPO_4, 1% SDS). Finally, the blotted

membrane was exposed to X-ray films at -80°C and detected by autoradiography.

2.4 RNA isolation and analysis

2.4.1 Isolation of total RNA from *P. putida*

Total RNA from *P. putida* S12 was isolated with the RNeasy Mini Kit and RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's instructions. Briefly, a single colony of *P. putida* S12 was used to inoculate 25 ml LB broth and grown overnight at 30°C. One ml of the overnight culture was used to inoculate 50 ml LB broth with or without 20% (v/v) toluene. The cultures were grown at 30°C until OD_{600} reached 1.0. At this point, 3 ml of culture (approximately $9x10^8$ cells) were taken for RNA isolation. The RNase-Free DNase Set (Qiagen) was used according to the manufacturer's recommendations to eliminate DNA contamination. Upon completion of the isolation procedure, the RNA sample was dissolved in 50 µl of RNase-free water and stored at -80°C. The ratio of A_{260}/A_{280} was used to quantify the RNA samples.

2.4.2 Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase (RT)-PCR was performed with 1 µg of RNA in a 20-µl reaction volume using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), and PCR amplification of the cDNA was performed with the Platinum *Taq*

DNA Polymerase High Fidelity (Invitrogen) as described above. The first-strand cDNA synthesis step was conducted at 55°C for 50 min. Following that, 2 µl of cDNA was used for PCR amplification, which had the same cycling conditions as described in section 2.3.1. Positive and negative controls were performed in all assays. Total Hela RNA and the sense and antisense control primers provided with the SuperScript III kit were used as the positive control. The negative controls were performed in the same way as the other reactions, except without the addition of the SuperScript III reverse transcriptase. The primers used for cDNA synthesis and PCR are listed in Table 2.3.

2.5 Test of survival frequency in response to toluene shocks

Single colonies of different *P. putida* strains were used to inoculate 5 ml of LB medium and grown overnight at 30°C. Kanamycin was added to a final concentration of 250 µg/ml to maintain the plasmids when necessary. On the following day, the cultures were diluted 1:25 and grown until the OD_{600} reached 1.0. After that, one ml of these regrown cultures was used to inoculate 50 ml of LB medium with 20% (v/v) toluene, or 20% (v/v) distilled water as a control. The cultures from the control group were taken immediately to spread LB plates, whereas the cultures from the toluene-shocked group were shaken vigorously (200 rpm) at 30°C for 30 min before being spread on LB plates. The cultures were diluted with 0.9% (w/v) saline, and 100µl of the suitable dilutions were plated on LB agar plates. The survival frequency was determined by measuring the

number of colony-forming units (cfu) on the LB plates after 20 h of incubation, and comparing the data between the test group and the control group.

2.6 Minimum inhibitory concentration (MIC) assays

The MIC assays were performed in LB medium by the microtiter broth dilution method (Handford 2005). Various toxic compounds and antibiotics, including sodium tungstate, nickel (II) chloride, gallic acid, indole, chloramphenicol, nalidixic acid, novobiocin, ampicillin, erythromycin, tetracycline, streptomycin, and polymyxin B, were used to test *P. putida* S12 and its derivatives. Stock solutions of drugs were prepared according to their solubilities listed in the Merck index (Budavari et al. 1996). The compounds were serially diluted into 96-well microtiter plates containing 100 µl of LB broth per well. After dilution, the concentration of each well was half that of the previous well. Single colonies of different *P. putida* strains were used to inoculate 5 ml of LB broth and grown overnight at 30°C. The overnight culture was diluted 1:25 in LB broth and incubated at 30°C until reaching a final OD_{600}=1.0. Five µl of the culture was used to inoculate each well of the microtiter plates containing antimicrobial solution. The microtiter plates were incubated on a shaker at 30°C for 20 h. MIC was determined by measuring OD_{600} with a Wallac Victor^2 multilabel counter (Perkin Elmer, Shelton, CT).

2.7 Protein analysis

2.7.1 Bacterial two-hybrid assays

The *srpR* and the *srpS* genes were PCR amplified with the primers indicated in Table 2.3, and then cloned into the bacterial two-hybrid vectors pKT25, pUT18, and pUT18C, respectively, using the restriction sites indicated in Table 2.2. The derivative constructs pKT25-*srpS*, pUT18-*srpR*, pUT18C-*srpR*, pKT25-*srpR*, pUT18-*srpS*, and pUT18C-*srpS*, were used in the bacterial two-hybrid assays to study the interaction between the SrpR and SrpS proteins. Four test groups (pKT25-*srpS* + pUT18-*srpR*, pKT25-*srpS* + pUT18C-*srpR*, pKT25-*srpR* + pUT18-*srpS*, and pKT25-*srpR* + pUT18C-*srpS*), one positive control group (pKT25-zip + pUT18C-zip), and one negative control group (pKT25 + pUT18) were conducted simultaneously in the assays. Two non-reverting adenylate cyclase deficient (*cya*) *E. coli* strains, BTH101 and DHM1, were both transformed with the plasmids in the test groups and the control groups. Transformants were plated on LB plates containing antibiotics (100 μg/ml ampicillin and 50 μg/ml kanamycin), IPTG and X-gal. The plates were incubated at 30°C, 37°C, or 42°C for up to 72 h, and checked for the color of the colonies.

2.7.2 In vitro glutathione S-transferase (GST) pull-down assays

2.7.2.1 Overexpression of GST-SrpR, His-SrpS, and His-ArpR

The *srpR* gene was PCR amplified from pJD101 using primers #32 and #33, which

contain engineered *Bam*HI and *Eco*RI sites, respectively. The amplified fragment was digested accordingly and cloned in-frame, downstream of the glutathione S-transferase gene in the plasmid pGEX-4T-1 (Amersham), resulting in the N-terminal GST-tag construct pGEX-*srpR*. The *srpS* gene was PCR amplified from pJD101 using primers #28 and #29, which contain engineered *Bam*HI and *Kpn*I sites, respectively. The amplified fragment was digested accordingly and cloned in-frame, downstream of the 6x histidine coding genes in the plasmid pQE31 (Qiagen), resulting in the N-terminal 6xHis-tag construct pQE31-*srpS*. Likewise, *arpR* was cloned into pQE31 with primers #30 and #31, and used as a negative control for the pull-down assay. *E. coli* BL21 was used to express GST and GST fusion proteins, and *E. coli* M15[pREP4] was used to express the 6xHis fusion proteins. To overexpress the fusion proteins, *E. coli* cells transformed with the appropriate plasmids were grown at 37°C in 2x YT broth until the OD_{600} reached 0.7, and IPTG was added to a final concentration of 0.05 mM to induce the expression of the fusion proteins. Incubation was continued for one additional hour at 30°C before the cells were harvested. A low IPTG concentration and short inducing time at a relatively low temperature was applied to reduce inclusion bodies and increase fusion protein solubility. After the induction, the cells were harvested by centrifugation at 7000 x g for 10 min and resuspended in 50 µl of ice-cold PBS buffer (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.3]) for each ml of culture that was centrifuged. Ten µl of these resuspended cells was collected for SDS-PAGE analysis

(section 2.7.2.3), and the rest of the cells were lysed by six cycles of 10-s sonication at 250 W with a 10-s cooling period using a Braun-Sonic 2000 Sonicator. Subsequently, the sonicates were centrifuged at 10000 x g for 15 min to remove insoluble material, and the supernatants were saved as the crude cell lysate samples. These total protein samples were quantified with a Bio-Rad protein assay kit (section 2.7.2.2), and stored at -80°C until being used in the pull-down assay.

2.7.2.2 Protein quantification

The total protein samples were quantified using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad) according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

2.7.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed by SDS-PAGE with slight modifications to the procedure described by Sambrook et al. (1989), using the Bio-Rad Mini-Protean 3 Electrophoresis Cell. The separating gel consisted of 12% (v/v) acrylamide (19:1 acrylamide: N,N'-methylenebisacrylamide [Bio-Rad]), 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.075% (w/v) ammonium persulfate (APS), and 0.08% tetramethyl ethylene diamine (TEMED). Once the separating gel solution was poured into the cast, dH_2O was added to cover the top of the separating gel, and the gel was allowed to polymerize for

20 min. Once the gel polymerized, the water on top was poured off, and the stacking gel solution was added. The stacking gel consisted of 5% (v/v) acrylamide (19:1 acrylamide: N,N'-methylenebisacrylamide [Bio-Rad]), 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.08% (w/v) APS and 0.2% (v/v) TEMED. The stacking gel was allowed to polymerize for 15 min before the protein samples were loaded. Prior to loading, the protein samples were combined with 5x SDS loading buffer (60 mM Tris-HCl [pH 6.8], 25% [v/v] glycerol, 2% [w/v] SDS, 14.4 mM β-mercaptoethanol, 0.1% [w/v] bromophenol blue) followed by denaturation at 95°C for 5 min. Electrophoresis was conducted in 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS) at a constant voltage of 200 V. The BenchMark Protein Ladder (Invitrogen) was used as the molecular weight marker. Following the electrophoresis, the gel was stained in Coomassie gel stain (0.1% [w/v] Coomassie Blue R-250, 45% [v/v] methanol, 10% [v/v] glacial acetic acid) for 10 min with gentle agitation. The gel was then rinsed with a few changes of dH_2O and destained for 2-16 h with gentle agitation in Coomassie gel destain (10% [v/v] methanol, 10% [v/v] glacial acetic acid). Finally, the gel was rinsed and stored in dH_2O, or saved as a digital picture file using an image scanner.

2.7.2.4 GST pull-down assays

GST pull-down assays were performed with modifications to the procedure described in the ProFound Pull-Down GST Protein:Protein Interaction Kit (Pierce

Biotechnology, Inc., Rockford, IL). A test group (GST-SrpR + His-SrpS) and three negative control groups (GST-SrpR + His-ArpR, GST + His-SrpS, and pure PBS buffer + His-SrpS) were conducted simultaneously in the pull-down assays. Preliminary experiments were performed to determine the concentrations of different cell lysate samples to be used, so that the test group and the control groups would have the same amount of GST-SrpR and GST, as well as the same amount of His-SrpS and His-ArpR. Formal pull-down assays were then performed accordingly. One ml of 0.83 mg/ml of the cell lysate containing GST-SrpR, one ml of 0.14 mg/ml of the cell lysate containing GST, and one ml of PBS buffer were individually incubated with 20 µl of 50% glutathione sepharose beads (Amersham) at 4°C for 1 h. The beads were then washed twice with 1 ml PBST (PBS + 0.05% [v/v] Tween-20) and once with 1 ml PBS. Following the wash steps, 1 ml of 0.22 mg/ml of the cell lysate containing His-SrpS or His-ArpR was added to the beads, and the samples were incubated with gentle agitation at 4°C for 2 h. Subsequently, the beads were washed twice with PBST and twice with PBS, and heated in SDS-PAGE loading buffer at 95°C for 5 min. Finally, the beads were briefly spun down, and the supernatant was separated on an SDS-PAGE gel and analyzed by Western blotting (section 2.7.2.5). The pull-down assays were repeated for three independent experimental rounds.

2.7.2.5 Western blot analysis

Proteins in unstained SDS-PAGE gels were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad) using the Bio-Rad Mini Trans-Blot Cell. The transfer sandwich was made of two pieces of sponge, two pieces of Whatmann 3MM filter paper, one piece of gel, and one piece of nitrocellulose membrane. The gels were transferred in 1x transfer buffer (25 mM Tris base, 0.2 M glycine, 20% [v/v] methanol) at 10 V overnight. Following transfer, the nitrocellulose membranes were stained with Ponceau S (Bio-Rad) to visualize the proteins, and the dye was then rinsed off with dH_2O before proceeding to the blocking step. The membranes were blocked with 3% (w/v) BSA in TBS buffer (10 mM Tris-Cl, 150 mM NaCl) for 1 h at room temperature.

For detection of the GST-tagged proteins, an immunodetection procedure was performed as described in the ProFound Far-Western GST-Protein:Protein Interaction Kit (Pierce). Briefly, the blocked membranes were washed six times (5 min each) with PBST buffer before being incubated with Anti-GST-Horseradish Peroxidase Antibody (final concentration of 5 ng/ml [Pierce]) for 1 h at room temperature. The membranes were then washed with PBST buffer again for six times (5 min each), and detected by a chemiluminescent method with 10 ml of the UnBlot Substrate Working Reagent (Pierce) and exposed to X-ray films. For detection of the 6xHis-tagged proteins, the procedure was conducted according to the QIA*express* Detection and Assay Handbook (Qiagen). Briefly, the blocked membranes were washed twice (10 min each) with

TBS-Tween/Triton buffer (20 mM Tris-Cl, 500 mM NaCl, 0.05% [v/v] Tween 20, 0.2% [v/v] Triton X-100) and once (10 min) with TBS buffer. Subsequently, the membranes were incubated with Penta-His Antibody (final concentration of 100 ng/ml [Qiagen]) for 1 h at room temperature. The same wash steps were then applied to the membranes as described above. After that, the membranes were incubated with the secondary antibody, which is peroxidase-conjugated goat anti-mouse IgG (final concentration of 16 ng/ml [Jackson ImmunoResearch Laboratories Inc., West Grove, PA]). After 1h incubation with secondary antibody, the membranes were washed four times (10 min each) with TBS-Tween/Triton buffer before incubated with 10 ml of the UnBlot Substrate Working Reagent (Pierce) and exposed to X-ray films.

Chapter 3: Results

3.1 Transcriptional analysis of the *srpRS-srpABC* gene cluster

The *srpABC* genes were shown previously to be polycistronic (Kieboom et al. 1998a). Since the transcriptional orientation of *srpRS* is opposite from that of *srpABC*, these two regulatory genes must not be in the same transcriptional unit as the three structural genes are. However, it was unclear whether *srpR* and *S* formed a single transcriptional unit. To study this, total RNA was isolated from the cells grown in the absence or presence of 20% (v/v) toluene, and RT-PCR assays were performed with primer pairs within *srpR* (primers #16 and #17), *srpS* (primers #1 and #2), and across *srpRS* (primers #16 and #2). The results showed that *srpR* and *srpS* were transcribed as a single transcriptional unit no matter whether toluene was present or not, and thus these two genes are polycistronic (Fig. 3.1).

P. putida DOT-T1E is another solvent-tolerant *P. putida* strain. The TtgGHI efflux pump in this strain is highly homologous (98% nucleotide sequence identity) to the SrpABC efflux pump in *P. putida* S12 (Rojas et al. 2001). The structural genes *ttgGHI* correspond to *srpABC*, and the two regulator genes *ttgV* and *ttgW* correspond to *srpS* and *srpR*, respectively. According to Rojas et al. (2003), the transcription start site of *ttgV* corresponds to the G indicated by "+1P_S" in Fig. 3.2(A), and the translation start codon of *ttgV* corresponds to the arrow indicated by "*srpS*" in Fig. 3.2(A). To determine if these sites were also accurate for the *srpRS* transcriptional unit, RT-PCR assays were performed with primers upstream (primer #20) and downstream (primers #18 and #19)

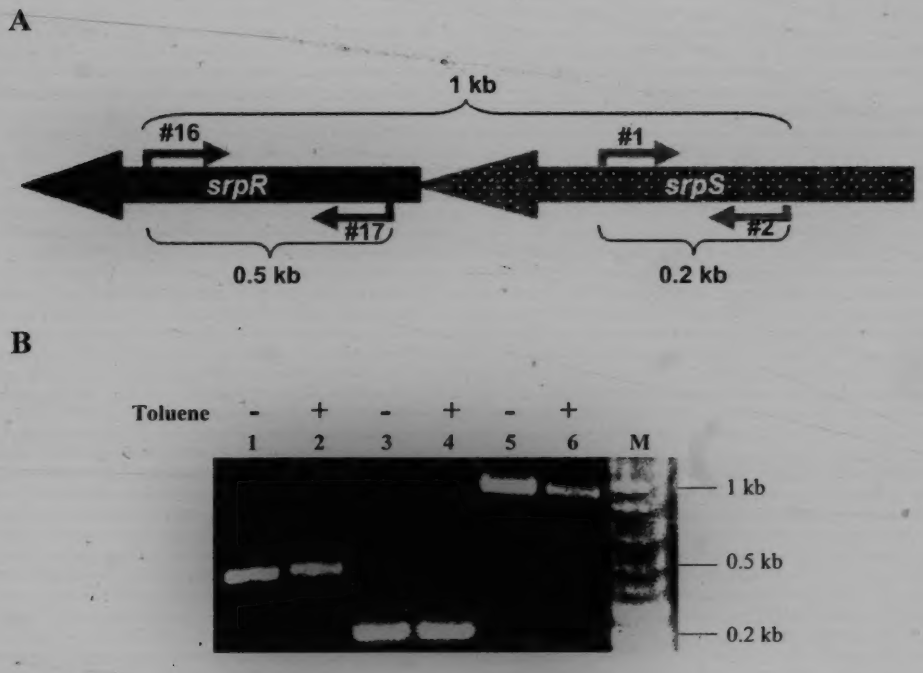


Figure 3.1. RT-PCR analysis of *srpRS* in *P. putida* S12. (A) RT-PCR primers and amplified region. Primers #16 and #17 were used to amplify *srpR*; primers #1 and #2 were used to amplify *srpS*; primers #16 and #2 were used to amplify *srpRS*. The expected fragment sizes are also shown. (B) RT-PCR result. Lanes 1&2, *srpR* amplification; lanes 3&4, *srpS* amplification; lanes 5&6, *srpR-srpS* amplification; lane M, molecular weight markers. RNA templates in lanes 1, 3 & 5 were isolated from the cells grown without toluene; RNA templates in lanes 2, 4 & 6 were isolated from the cells grown with 20% (v/v) toluene.

Figure 3.2. Determination of the transcription start site of the *srpS* gene and *srpA* gene in *P. putida* S12. (A) Overlap region of the *srpS* and *srpA* promoters. The direction of transcription of *srpS* and *srpA* are indicated by arrows followed by "*srpS*" or "*srpA*". The transcription start site for *srpS* and *srpA* are designated as +1Ps and $+1P_{A}$, respectively. The -10 and -35 positions of each promoter are indicated. The primers used in RT-PCR assays are underlined, with their names indicated above or below. (B) RT-PCR result for confirming the transcription start site of *srpS*. Lanes 1&4, amplification with primer #20 (expected size, 760 bp); lanes 2&5, amplification with primer #18 (expected size, 694 bp); lanes 3&6, amplification with primer #19 (expected size, 524 bp). (C) RT-PCR result for confirming the transcription start site of *srpA*. Lanes 1&4, amplification with primer #23 (expected size, 346 bp); lanes 2&5, amplification with primer #22 (expected size, 358 bp); lanes 3&6, amplification with primer #24 (expected size, 371 bp); lane M, molecular weight markers. RNA templates in lanes 1, 2 & 3 of (B) and (C) were isolated from the cells grown without toluene; RNA templates in lanes 4, 5 & 6 were isolated from the cells grown with 20% (v/v) toluene.

A

CATGATCGAGGCTGCTCTGGCGATGACCTGGATGCCCCCGGCCTTGCCAACATTTTC
GTACTAGCTCCGACGAGACCGCTACTGGACCTACGGGGGCCGGAACGGTTGTAAAAG

#19

ATCTGATTGGTTCATATCTTTCCTCTGCGCGTACGGCTGCGACCTATCACATTGGCC
TAGACTAACCAAGTATAGAAAGGAGACGCGCATGCCGACGCTGGATAGTGTAACCGG

srpS

GCCACTGTAGAGCTCTCTGCCTCCGCTAGCCAGCCACACATGGCGAACGCCCCAATC
CGGTGACATCTCGAGAGACGGAGGCGATCGGTCGGTGTGTAGCGCTTGCGGGGTTAG

-35P$_A$ #24

GGTAAACCTCGACCAACGGCGGCTCAGGCTTGCTTGCATCGAGAGTATCGCATAATG
CCATTTGGAGCTGGTTGCCGCCGAGTCCGAACGAACGTAGCTCTCATAGCGTATTAC

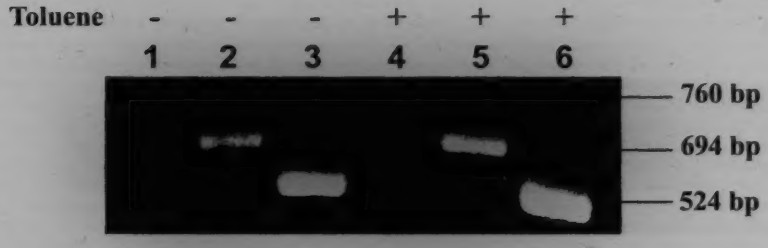
#18 +1P$_S$ -10P$_S$

-10P$_A$ #22 +1P$_A$ #23 *srpA*

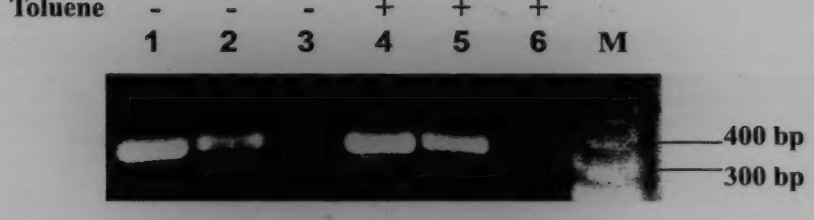
GTAGACTCTACCGCATTACGATTCAGCAATAGCCCCCGTCATG
CATCTGAGATGGCGTAATGCTAAGTCGTTATCGGGGGCAGTAC

-35P$_S$ #20

B



C



from $+1P_S$. Total RNA samples isolated from the cells grown with or without 20% (v/v) toluene were used as templates, and the first-strand cDNA of *srpS* was amplified with primer #3. The following PCR amplification was done by adding primers #20, #18, and #19, respectively. Amplification was achieved when primers downstream from +1Ps were used, and no band was obtained with the primer upstream from +1Ps (Fig. 3.2[B]). This result supports the hypothesis that the G indicated by +1Ps in Fig. 3.2(A) is the correct transcription start site of *srpS* both in the presence and absence of toluene.

Likewise, the transcription start site of the *srpABC* operon was determined. According to Guazzaroni et al. (2004), the putative transcription start site of *srpA* in *P. putida* S12 is the A indicated by "$+1P_A$" in Fig. 3.2(A). To confirm this putative site, RT-PCR assays were performed with primers upstream of $+1P_A$ (primer #24), covering $+1P_A$ (primer #22), and downstream of $+1P_A$ (primer #23). Amplification was only achieved by using primer #22 or primer #23 (Fig. 3.2[C]), supporting the hypothesis that the $+1P_A$ in Fig. 3.2(A) is the correct transcription start site of the *srpABC* operon.

3.2 Discovery and characterization of a novel insertion sequence within the *srpS* gene

3.2.1 Discovery of a novel insertion sequence within *srpS*

Wery et al. (2001) reported that 93% of the *P. putida* S12 cells that survived a 1%

(v/v) toluene shock carried an extra copy of the 2.6-kb insertion sequence IS*S12* within the *srpS* gene. I therefore carried out an experiment (Fig. 3.3) similar to the one described by Wery et al. A single colony of *P. putida* S12 was used to inoculate 25 ml LB broth and grown overnight at 30°C. One ml of the overnight culture was used to inoculate 50 ml of LB broth containing 1% (v/v) toluene. This shock dose of toluene killed most of the cells (approximately 99.99%) within 30 min. After shaken (200 rpm) at 30°C for 24 h, a new culture had grown up, and total DNA was isolated from it to perform PCR. Primers #1 and #2, the region between which includes the insertion site of IS*S12*, were used to amplify the DNA sample (Fig. 3.4[A]). Interestingly, the PCR result only showed a band with the original size on *srpS* (data not shown), indicating that IS*S12* did not appear in this region as Wery et al. claimed. Then I tried a more rigorous condition of a 20% (v/v) toluene shock on the bacterial culture. Although the aqueous solubility of toluene is very limited, it has been reported that the toxic effect of toluene increases with increasing toluene concentrations, even at concentrations above its maximum aqueous solubility limit (Zahir 2005). It was proposed that the addition of higher concentrations of toluene in a shaking flask increases the contact frequency between the cells and the toluene phase, resulting in more cell death (Zahir 2005). Thus 20% (v/v) toluene is a harsher selective condition than 1% (v/v) toluene. However, with this condition, I still could not find any insertion sequences within the region between primers #1 and #2 (Fig. 3.4[B], lane 1). On the other hand, when another primer pair #3

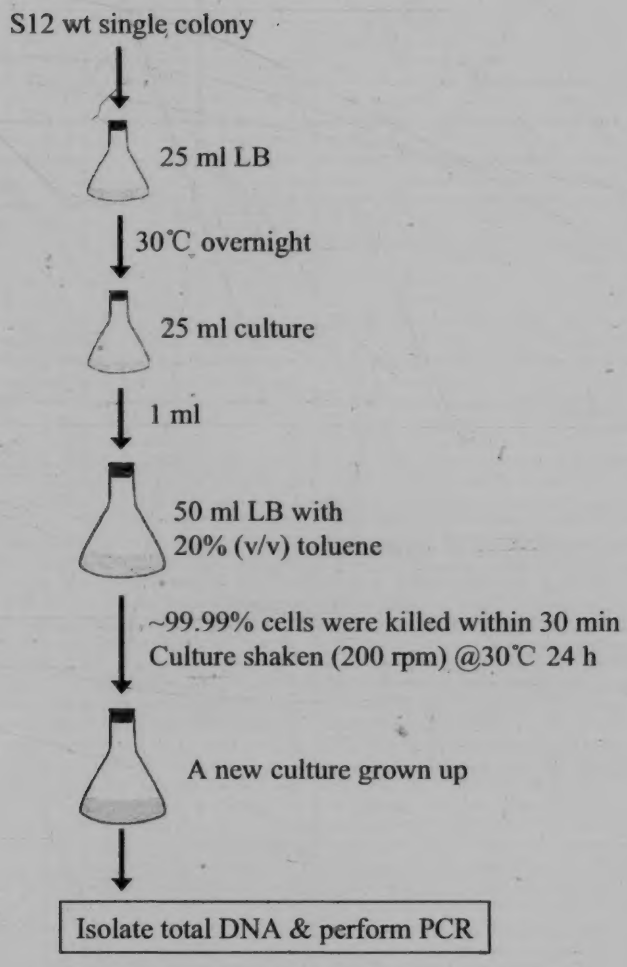
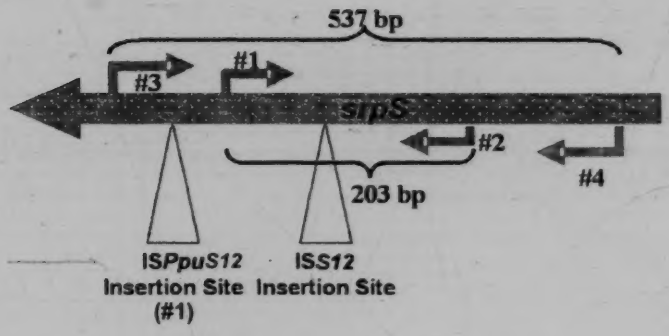

S12 wt single colony
25 ml LB
30℃ overnight
25 ml culture
1 ml
50 ml LB with
20% (v/v) toluene
~99.99% cells were killed within 30 min
Culture shaken (200 rpm) @30℃ 24 h
A new culture grown up
Isolate total DNA & perform PCR

Figure 3.3. Procedure for the detection of insertion sequences within *srpS* in the cells that survived high doses of toluene. See description in the text for details.

A



B

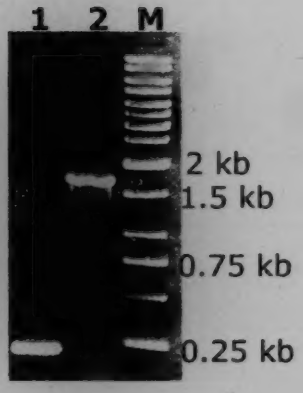


Figure 3.4. Discovery of the new insertion sequence IS*PpuS12* within the *srpS* gene in *P. putida* S12 by PCR. (A) Location of the PCR primers, sizes of the amplified regions, and the insertion sites of IS*S12* and IS*PpuS12*. (B) The products resulting from the PCR reaction were separated on a 0.7% (w/v) agarose gel. Lane 1, the region amplified with primers #1 and #2; lane 2, the region amplified with primers #3 and #4; lane M, molecular weight markers.

and #4, which covers a larger region on *srpS* than do primers #1 and #2 (Fig. 1A), was used to amplify the DNA sample from the cells that survived the 20% (v/v) toluene shock, the PCR result showed a band of 1.7 kb, much larger than the expected size of 537 bp (Fig. 3.4[B], lane 2). This result indicated that there must be a fragment inserted into the region between primers #1 and #3, or the region between primers #2 and #4. Later, the DNA sequencing results confirmed this hypothesis, showing that there was a 1.2-kb insertion sequence located in the region between primers #1 and #3. Following sequence analysis, this new insertion sequence designated as IS*PpuS12*, was determined to contain no homology to IS*S12*, and its insertion site in *srpS* is different from that of IS*S12* (Fig. 3.4[A]).

Subsequently, I went on to test if all the cells surviving the 20% (v/v) toluene shock carry an insertion sequence within the repressor protein gene *srpS* (Fig. 3.5). To ensure that the cells used for total DNA extraction were genetically homogenous, an aliquot of the culture incubated with 20% (v/v) toluene for 24 h was spread on LB agar plates, which were then incubated at 30°C in a sealed glass chamber with saturated toluene vapour in it. After another 24-48 h, single colonies were obtained on the LB plates and each colony was amplified by incubating on independent LB plates in the sealed chamber with saturated toluene vapour. Thirty-three separate cultures grown up from different single colonies were used for total DNA extraction, and then PCR amplified with primers #8 and #9, which encompass the entire coding sequence of *srpR* and *srpS*.

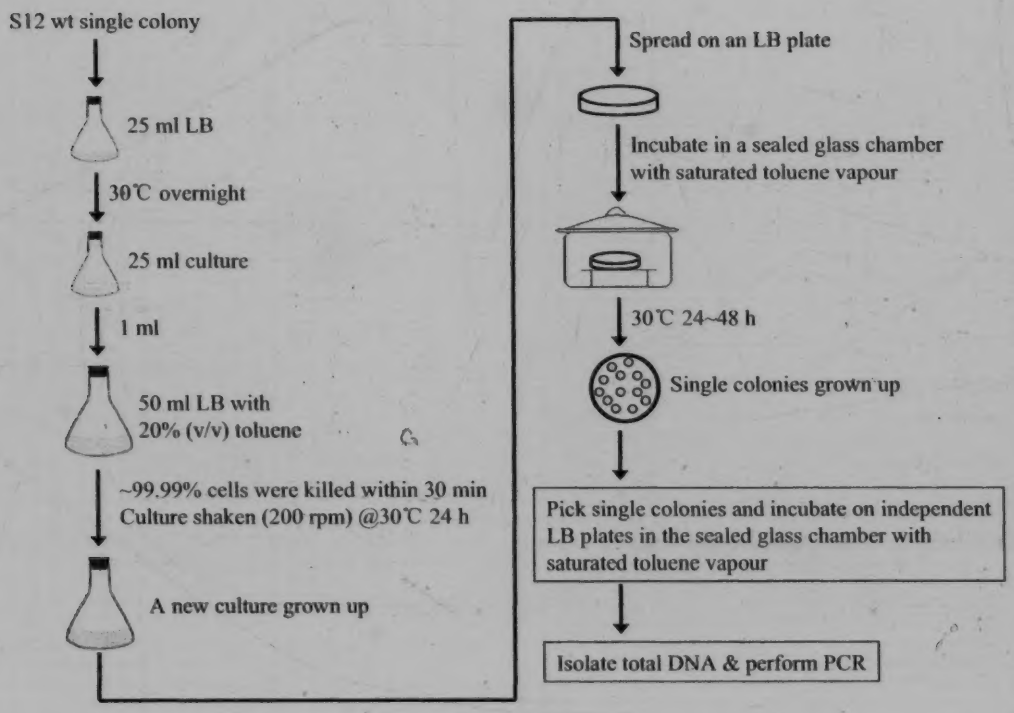

S12 wt single colony
25 ml LB
30℃ overnight
25 ml culture
1 ml
50 ml LB with
20% (v/v) toluene
~99.99% cells were killed within 30 min
Culture shaken (200 rpm) @30℃ 24 h
A new culture grown up
Spread on an LB plate
Incubate in a sealed glass chamber
with saturated toluene vapour
30℃ 24~48 h
Single colonies grown up
Pick single colonies and incubate on independent
LB plates in the sealed glass chamber with
saturated toluene vapour
Isolate total DNA & perform PCR

Figure 3.5. Procedure for the selection of genetically clonal cells that survived the 20% (v/v) toluene shock. See description in the text for details.

Among these different samples, 22 of them carried no insertion sequences at all within the amplified region, one of them showed IS*S12* located in the same site as discovered by Wery et al. (2001), and the other ten samples were found to have IS*PpuS12*. Further investigation by PCR and DNA sequencing on the ten samples that carried IS*PpuS12* showed two different insertion sites of IS*PpuS12*: one (#1 IS site) located between primers #1 and #3 (6/10 samples), and the other (#2 IS site) located downstream of primer #3 but still within *srpS* (4/10 samples). These results suggest that only 1/3 of the cells that survived the toluene shock carried an insertion sequence within the pump-repressor gene *srpS*, whereas the other 2/3 of the cells did not need the *srpS* mutation to survive the high-dose toluene shock.

3.2.2 Characterization of IS*PpuS12*

DNA sequencing of IS*PpuS12* showed that it is a 1179-bp insertion sequence with a pair of 6-bp inverted repeats (IR-U / IR-D) at its terminals (Fig. 3.6). As mentioned above, two different insertion points of IS*PpuS12* were found in the *srpS* gene (Fig. 3.7). Interestingly, the insertion of IS*PpuS12* at the #1 and the #2 IS sites generates a pair of 7-bp and a pair of 4-bp target site duplication direct repeats, respectively. Only one open reading frame was discovered in IS*PpuS12*, putatively encoding a protein of 326 amino acids. The nucleotide sequence of IS*PpuS12* has 100% identity with part of *P. aeruginosa* serotype O15-ATCC putative O-antigen biosynthesis gene cluster, and the

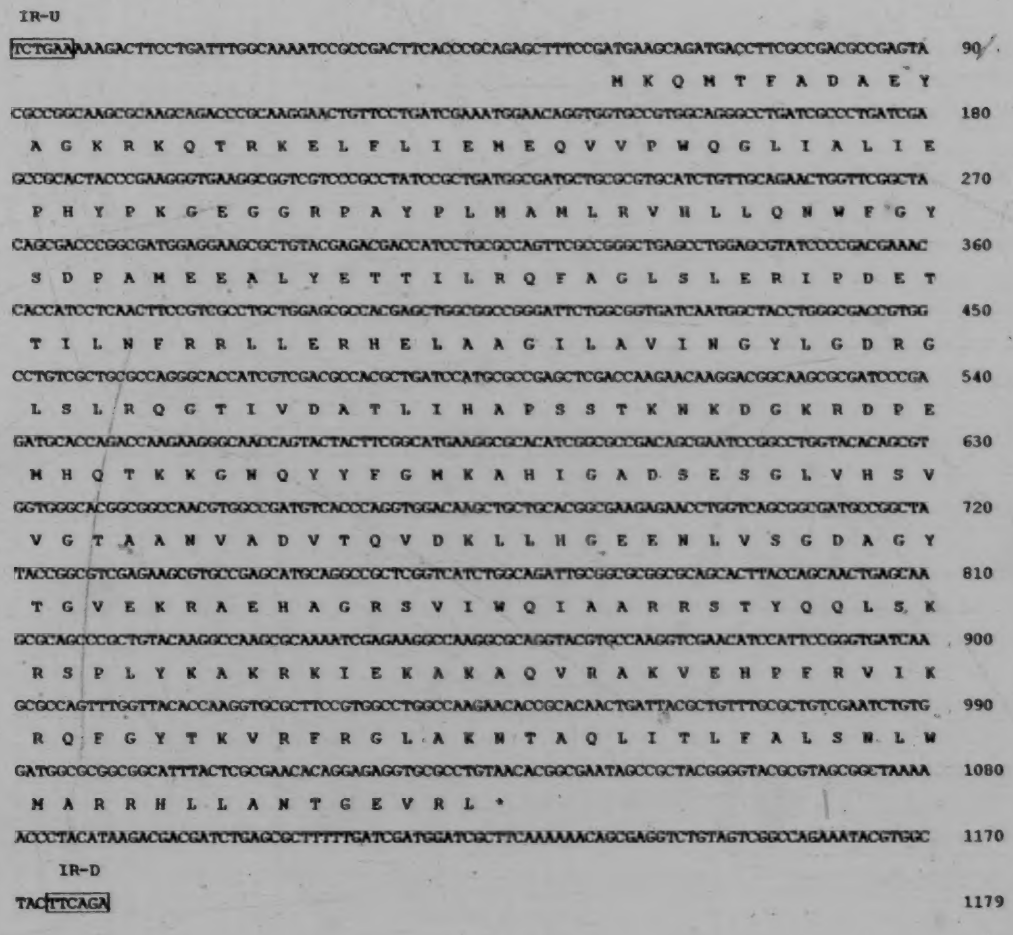

IR-U
TCTGAAAAAGACTTCCTGATTTGGCAAAATCCGCCGACTTCACCCGCAGAGCTTTCCGATGAAGCAGATGACCTTCGCCGACGCCGAGTA 90
M K Q M T F A D A E Y
CGCCGGCAAGCGCAAGCAGACCCGCAAGGAACTGTTCCTGATCGAAATGGAACAGGTGGTGCCGTGGCAGGGCCTGATCGCCCTGATCGA 180
A G K R K Q T R K E L F L I E M E Q V V P W Q G L I A L I E
GCCGCACTACCCGAAGGGTGAAGGCGGTCGTCCCGCCTATCCGCTGATGGCGATGCTGCGCGTGCATCTGTTGCAGAACTGGTTCGGCTA 270
P H Y P K G E G G R P A Y P L M A M L R V H L L Q N W F G Y
CAGCGACCCGGCGATGGAGGAAGCGCTGTACGAGACGACCATCCTGCGCCAGTTCGCCGGGCTGAGCCTGGAGCGTATCCCCGACGAAAC 360
S D P A M E E A L Y E T T I L R Q F A G L S L E R I P D E T
CACCATCCTCAACTTCCGTCGCCTGCTGGAGCGCCACGAGCTGGCGGCCCGGATTCTGGCGGTGATCAATGGCTACCTGGGCGACCGTGG 450
T I L N F R R L L E R H E L A A G I L A V I N G Y L G D R G
CCTGTCGCTGCGCCAGGGCACCATCGTCGACGCCACGCTGATCCATGCGCCGAGCTCGACCAAGAACAAGGACGGCAAGCGCGATCCCGA 540
L S L R Q G T I V D A T L I H A P S S T K N K D G K R D P E
GATGCACCAGACCAAGAAGGGCAACCAGTACTACTTCGGCATGAAGGCGCACATCGGCGCCGACAGCGAATCCGGCCTGGTACACAGCGT 630
M H Q T K K G N Q Y Y F G M K A H I G A D S E S G L V H S V
GGTGGGCACGGCGGCCAACGTGGCCGATGTCACCCAGGTGGACAAGCTGCTGCACGGCGAAGAGAACCTGGTCAGCGGCGATGCCGGCTA 720
V G T A A N V A D V T Q V D K L L H G E E N L V S G D A G Y
TACCGGCGTCGAGAAGCGTGCCGAGCATGCAGGCCGCTCGGTCATCTGGCAGATTGCGGCGCGGCGCAGCACTTACCAGCAACTGAGCAA 810
T G V E K R A E H A G R S V I W Q I A A R R S T Y Q Q L S K
GCGCAGCCCGCTGTACAAGGCCAAGCGCAAAATCGAGAAGGCCAAGGCGCAGGTACGTGCCAAGGTCGAACATCCATTCCGGGTGATCAA 900
R S P L Y K A K R K I E K A K A Q V R A K V E H P F R V I K
GCGCCAGTTTGGTTACACCAAGGTGCGCTTCCGTGGCCTGGCCAAGAACACCGCACAACTGATTACGCTGTTTGCGCTGTCGAATCTGTG 990
R Q F G Y T K V R F R G L A K N T A Q L I T L F A L S N L W
GATGGCGCGGCGGCATTTACTCGCGAACACAGGAGAGGTGCGCCTGTAACACGGCGAATAGCCGCTACGGGGTACGCGTAGCGGCTAAAA 1080
M A R R H L L A N T G E V R L *
ACCCTACATAAGACGACGATCTGAGCGCTTTTTGATCGATGGATCGCTTCAAAAAACAGCGAGGTCTGTAGTCGGCCAGAAATACGTGGC 1170
IR-D
TACTTCAGA 1179

Figure 3.6. Nucleotide sequence of IS*PpuS12* from *P. putida* S12. The deduced amino acid sequence of the encoded protein is shown below the nucleotide sequence. Upstream and downstream inverted repeat sequences (IR-U, IR-D) are boxed. The termination codon is indicated with an asterisk.

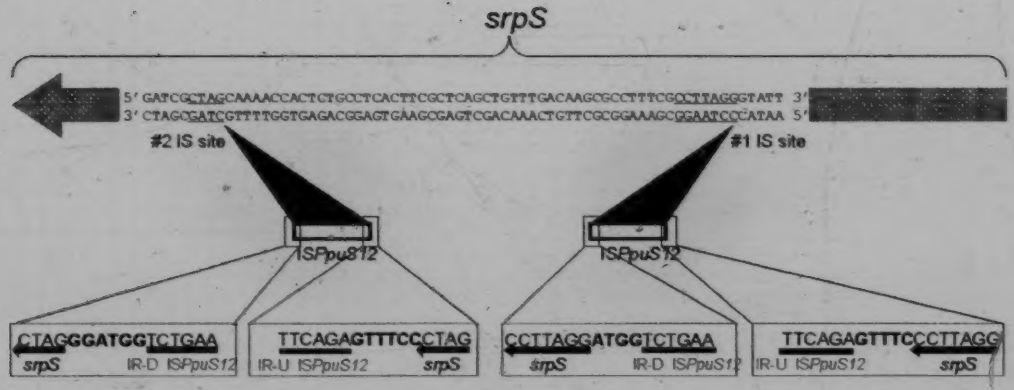


Figure 3.7. Schematic diagram of the insertion sites of IS*PpuS12* in the *srpS* gene in *P. putida* S12TS. The two black triangles indicate the two different insertion points of IS*PpuS12* in *srpS*. The sequences of the upstream and downstream terminal IR-U and IR-D, as well as the sequences adjacent to these terminal repeats, have been marked with boxes. The nucleotides not belonging to either *srpS* or IS*PpuS12* are shown in boldface.

ORF from this IS*PpuS12* orthologue is transcribed in the opposite direction to that of the *P. aeruginosa* chromosomal gene cluster. The protein putatively encoded by the ORF from IS*PpuS12* has very high homology (identities from 93% to 61%) with transposase proteins, mostly belonging to the IS*5* family of insertion sequences, from various bacterial species (Table 3.1).

3.2.3 Distribution of IS*PpuS12* in *P. putida* S12

Similar to Wery et al.'s experiment (2001), I determined the distribution of the new insertion sequence IS*PpuS12* over the genome of wild-type *P. putida* S12 and the mutant strain carrying IS*PpuS12* within *srpS* (designated as *P. putida* S12TS [toluene shock]). For this purpose, total DNA was isolated from both *P. putida* S12 and *P. putida* S12TS. The DNA samples were respectively digested with *Pst*I or *Eco*RI, which are restriction endonuclease that do not cut in the DNA of IS*PpuS12*. Subsequently, the DNAs were used to perform a Southern hybridization analysis with an internal 352-bp DNA probe made from IS*PpuS12* with primers #6 and #7 (Fig. 3.8). Lanes 1 and 3 show the results from wild-type *P. putida* S12; lanes 2 and 4 show the results from *P. putida* S12TS. From each of the wild-type samples, there are three visible hybridizing DNA bands, indicating that at least three copies of IS*PpuS12* are distributed over the genome of wild-type *P. putida* S12. On the other hand, one extra hybridizing DNA fragment appears in each sample from *P. putida* S12TS, indicating that the transposition of IS*PpuS12* occurs by a

Table 3.1. Amino acid sequence identity between the putative protein encoded by the ORF from IS*PpuS12* and homologous transposase proteins from different organisms

	Identity (%)	Organism	Accession number
Orf IS*PpuS12*			
IS*5* family transposase	93	*Pseudomonas resinovorans*	NP_758545
IS*Pssy*, transposase	93	*Bordetella petrii*	YP_001632795
IS*1384* transposase	92	*P. putida*	AAC98743
IS*Pst5* transposase	92	*Pseudomonas stutzeri*	AAX14063
IS*5* family transposase	91	*P. aeruginosa*	ZP_00972095
IS*Psy2* transposase	90	*Pseudomonas syringae*	YP_275487
IS*4* family transposase	90	*Azotobacter vinelandii*	ZP_00415717
IS*5* family transposase	89	*Yersinia enterocolitica*	CAE46781
IS*1479* transposase	65	*Xanthomonas campestris*	YP_243093
IS*5* family transposase	62	*Xanthomonas oryzae*	YP_201728
IS*5* family transposase	62	*Achromobacter xylosoxidans*	YP_195864
IS*4* family transposase	62	*Burkholderia vietnamiensis*	YP_001109709
IS*BPH* transposase	61	*Achromobacter denitrificans*	NP_990893

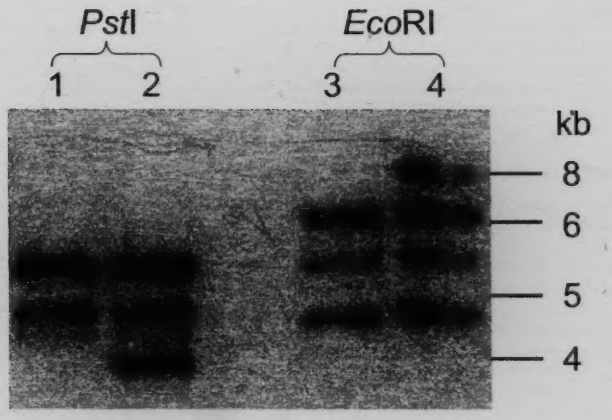


Figure 3.8. Southern hybridization analysis of total DNA from *P. putida* S12 and *P. putida* S12TS. The DNA samples were digested with *Pst*I (lanes 1&2) or *Eco*RI (lanes 3&4), and analyzed by Southern hybridization with a 352-bp DNA probe made from IS*PpuS12*. Lanes 1 and 3 are the results from wild-type *P. putida* S12; lanes 2 and 4 are the results from *P. putida* S12TS.

replicative mechanism.

3.2.4 Survival frequency of *P. putida* S12TS in response to toluene shocks

Wery et al. (2001) studied the survival frequency of wild-type S12 and the *srpS*::IS*S12* mutant S12PT in response to sudden exposure to toluene shocks, and concluded that the increased survival rate of S12PT after a sudden toluene shock dropped back to the wild-type level only if both *srpR* and *S* were complemented. However, I obtained different results by performing a similar experiment. The plasmid pBBR1MCS-2 used in this experiment is a broad host-range cloning vector carrying a kanamycin-resistance marker. This plasmid has been shown to replicate in a variety of bacterial hosts including *E. coli* and *P. putida* (Kovach et al. 1995). pBBR1MCS-2 was used for cloning of *srpR*, *srpS*, and *srpRS* as follows: DNA fragments containing the *srpRS* genes and the upstream and downstream region were amplified by PCR, using primers #8 and #9, and cloned into plasmid pCR2.1-TOPO, which is the TOPO TA cloning vector for direct insertion of PCR products (Invitrogen). The recombinant plasmid pCR2.1-*srpRS* was then used as the template to amplify the fragments containing the *srpR*, *srpS*, and *srpRS* genes, using primers #12 and #13, #14 and #15, #14 and #13, respectively. All of these three amplified fragments were flanked by a restriction site of *Bam*HI and *Eco*RI, and were ligated into pBBR1MCS-2 cut with the same enzymes. The corresponding constructs were designated as pBBR1-2-*srpR*,

pBBR1-2-*srpS*, and pBBR1-2-*srpRS*, respectively. These three constructs were transformed into *E. coli* DH5α for storage and propagation, and finally electroporated into *P. putida* S12TS for the survival frequency complementation experiment.

The survival frequency assay was conducted as described in Materials and Methods (section 2.5), and the results are listed in Table 3.2. It was shown that the *srpS*::IS*PpuS12* mutant S12TS had a 17000-fold increased survival frequency in the presence of a 20% (v/v) toluene shock in comparison with wild-type S12, and this was not affected by the introduction of the empty vector pBBR1MCS-2. Interestingly, both S12TS (pBBR1-2-*srpS*) and S12TS (pBBR1-2-*srpRS*) showed survival frequencies close to the wild-type value, which suggests that the survival frequency of S12TS returns to the wild-type level as long as *srpS* is complemented. *srpR* did not seem to affect the survival frequency, as the value remained the same as S12TS (5%) when S12TS was complemented with *srpR* alone.

Further investigation was performed on the 0.0003% of the wild-type cells that survived after being exposed to 20% (v/v) toluene for 30 min. Total DNA was isolated from 9 individual colonies and analyzed by PCR with primers #8 and #9. The results showed that 4/9 of the samples had IS*PpuS12* within *srpS*, while the other 5/9 had intact *srpS* (data not shown). This is consistent with our results described in section 3.2.1, and again suggests that more than half of the S12 cells do not need the *srpS* mutation to tolerate toluene shocks.

Table 3.2. Survival frequency of different *P. putida* strains in response to a 20% (v/v) toluene shock[a]

P. putida strain[b]	Survival frequency (%)
S12	0.0003 ± 0.0002
S12TS	5 ± 1.5
S12TS(pBBR1MCS-2)	5 ± 2.6
S12TS(pBBR1-2-*srpR*)	5 ± 2.3
S12TS(pBBR1-2-*srpS*)	0.0003 ± 0.0002
S12TS(pBBR1-2-*srpRS*)	0.0002 ± 0.0001

[a] All assays were repeated three times from three separate cultures. The given values are the mean of triplicate measurements.

[b] The names of the introduced plasmids are parenthesized.

The differences between the findings obtained in this study and the results described in Wery et al.'s publication (2001) are listed in Table 3.3.

3.3 Effect of various toxic compounds and antibiotics on *P. putida* S12 and its derivatives

The BaeSR two-component regulatory system, one of the envelope stress response regulatory systems in *E. coli*, is responsible for the regulation of the MdtABC efflux pump (Baranova and Nikaido 2002; Nagakubo et al. 2002; Raffa and Raivio 2002). Zhou et al. (2003) reported that the *E. coli* strains lacking the BaeSR system showed increased sensitivity to several toxic compounds, such as sodium tungstate, nickel (II) chloride, and gallic acid, suggesting that these compounds could be substrates of the MdtABC efflux pump. Recently, another toxic compound, indole, which can affect the integrity of the cell membrane, was demonstrated to be an inducer of the multidrug efflux systems in *E. coli* and *P. putida* (Guazzaroni et al. 2005; Hirakawa et al. 2005). To determine the effect of the above compounds on the SrpABC efflux pump, minimum inhibitory concentration (MIC) assays were performed with *P. putida* S12 and its derivatives. In addition, various antibiotics were also included in the MIC assays. Although previously described as an efflux system that can only be induced by organic solvents (Kieboom et al. 1998b), some evidence in our lab and other labs (Rojas et al. 2001) suggested that the SrpABC efflux pump could also participate in antibiotic extrusion.

Table 3.3. Results described in Wery et al.'s publication (2001) versus results obtained in this study

	Wery et al.'s description (2001)	Discoveries obtained in this study
The culture grown up after 24 h incubation of the cells that survived 1% (v/v) toluene shock	Always found the mutant with *srpS*::IS*S12*	Did not find any *srpS* mutant
The culture grown up after 24 h incubation of the cells that survived 20% (v/v) toluene shock	N/A	11/33 (33%) cells had IS within *srpS*; 22/33 (67%) cells had no IS within *srpS*
The cells that survived after being exposed to a toluene shock for 30 min	14/15 (~93%) individual colonies had IS*S12* within *srpS*; 1/15 (~7%) individual colonies had no IS within *srpS*	4/9 (44%) individual colonies had IS*PpuS12* within *srpS*; 5/9 (56%) individual colonies had no IS within *srpS*
Conclusion of the survival frequency assays	Both *srpS* and *srpR* are needed for effective repression of *srpABC*	Only *srpS* is the repressor protein gene

Another purpose of the MIC assays was to further determine the function of SrpS. Although the *srpS* gene is located closely upstream from the structural genes of the SrpABC efflux pump, it is possible that the SrpS protein is involved in a global regulatory system and represses not only SrpABC but also other efflux systems in *P. putida* S12. Therefore, to further determine the function of SrpS, the SrpABC efflux pump in *P. putida* S12TS was inactivated by homologous recombination, creating an *srpS-srpB* double mutant, which was also included in the MIC assays.

3.3.1 Construction of the *srpS-srpB* double mutant

P. putida JK1 is a solvent-sensitive mutant of *P. putida* S12 derived previously (Kieboom et al. 1998a), with its *srpB* gene interrupted by a plasposon Tn*Mod*-KmO (Dennis and Zylstra 1998). The plasmids pJD101 and pJD102 (Fig. 3.9) are two clones obtained by *Bam*HI and *Pst*I digestion and self-ligation of chromosomal DNA from *P. putida* JK1 (Kieboom et al. 1998a). To create a new plasmid suitable for homologous recombination, both pJD101 and pJD102 were digested by *Eco*RV and *Bam*HI. Afterwards, the fragment containing Tn*Mod*-KmO in pJD101 and the fragment without Tn*Mod*-KmO in pJD102 were ligated together. The newly-constructed plasmid, designated pJD203, was electroporated into *P. putida* S12TS to replace the wild-type copy of *srpB* with the gene mutated by the plasposon, resulting in the *srpS-srpB* double mutant *P. putida* S12DM. The IS mutation in *srpS* and the plasposon mutation in *srpB*

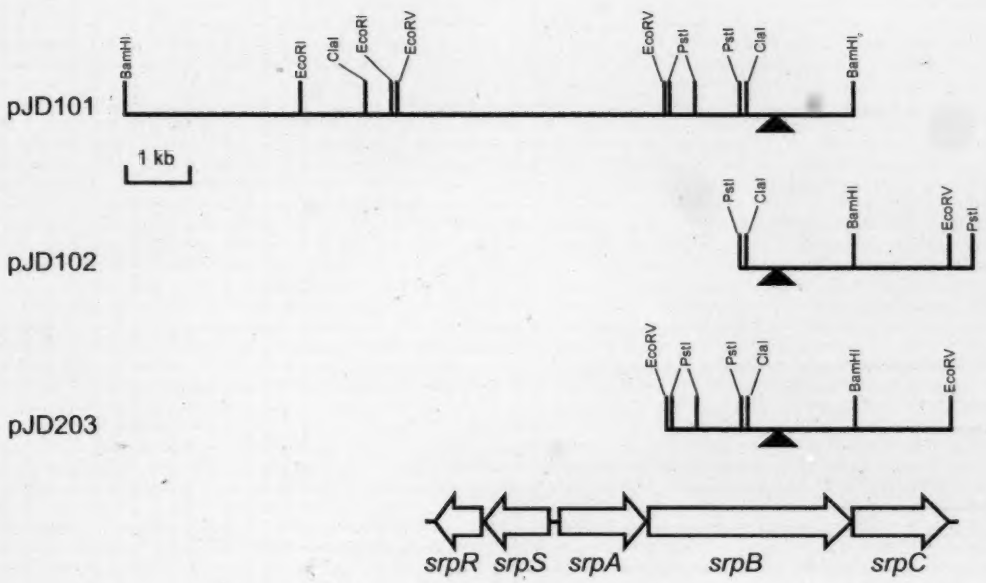


Figure 3.9. Restriction maps of pJD101 and pJD102, and the newly-constructed plasmid pJD203. A schematic diagram of the *srpRS-srpABC* gene cluster is shown at the bottom, in proportion to the restriction maps and showing the positions of the genes relative to the restriction sites. The triangles in the maps indicate the location of the inserted plasposon Tn*Mod*-KmO.

were confirmed by PCR with primers #3 & #2 and primers #10 & #11, respectively (Fig. 3.10).

3.3.2 MIC assays on *P. putida* S12 and its derivatives

The MIC assays were performed according to the procedure described in Materials and Methods (section 2.6), with wild-type *P. putida* S12 and three of its derivatives S12TS, JK1, and S12DM. As shown in Table 3.4, wild-type *P. putida* S12 and the three mutant strains have the same sensitivity to sodium tungstate, nickel (II) chloride, and gallic acid, indicating that these compounds are not substrates of the SrpABC efflux pump. However, the wild-type strain and the IS mutant S12TS showed a twofold stronger tolerance to indole than did JK1 and the double mutant S12DM, suggesting that indole is a substrate of the SrpABC efflux pump. Since this experiment has been repeated over five independent trials with the same trend, the twofold difference observed appears to be real and reliable. On the other hand, all the four test strains showed the same MIC for ampicillin, erythromycin, tetracycline, streptomycin, and polymyxin B, indicating that the SrpABC efflux pump is not responsible for the extrusion of these antibiotics. Nevertheless, S12 and S12TS were twofold more resistant to nalidixic acid and novobiocin than were JK1 and S12DM, suggesting that both of these antibiotics are substrates of the SrpABC efflux pump. More interestingly, the MIC result for wild-type S12 in chloramphenicol was higher than JK1 and S12DM, but lower

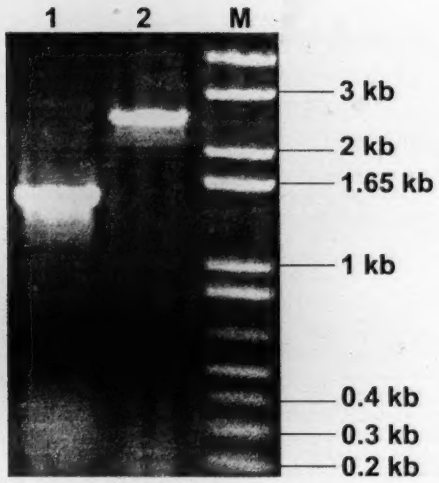


Figure 3.10. Confirmation of the *srpS*::IS*PpuS12* and *srpB*::Tn*Mod*-KmO mutations in the *srpS-srpB* double mutant *P. putida* S12DM by PCR. Chromosomal DNA was isolated from *P. putida* S12DM, and used as the template in PCR. Primers #3 and #2 were used to amplify a region on *srpS*, including the insertion site of IS*PpuS12*; primers #10 and #11 were used to amplify a region on *srpB*, including the insertion site of Tn*Mod*-KmO. The PCR products were separated on a 0.7% (w/v) agarose gel. Lane 1, a 1.5-kb DNA fragment amplified by primers #3 and #2 (0.3 kb on *srpS* plus 1.2 kb of IS*PpuS12*); lane 2, a 2.5-kb DNA fragment amplified by primers #10 and #11 (0.4 kb on *srpB* plus 2.1 kb of Tn*Mod*-KmO); lane M, molecular weight markers.

Table 3.4. MICs of various toxic compounds and antibiotics for *P. putida* S12 and its derivatives[a]

Chemical	MIC for strain:			
	S12	S12TS	JK1	S12DM
Sodium tungstate (mg/ml)	100	100	100	100
Nickel (II) chloride (mg/ml)	2.5	2.5	2.5	2.5
Gallic acid (mg/ml)	5	5	5	5
Indole (μg/μl)	2400	2400	1200	1200
Chloramphenicol (μg/μl)	512	1024	256	256
Nalidixic acid (μg/μl)	512	512	256	256
Novobiocin (μg/μl)	1024	1024	512	512
Ampicillin (μg/μl)	256	256	256	256
Erythromycin (μg/μl)	1024	1024	1024	1024
Tetracycline (μg/μl)	16	16	16	16
Streptomycin (μg/μl)	256	256	256	256
Polymyxin B (μg/μl)	2	2	2	2

[a] All assays were repeated over five independent trials with the same trend. Representative results are shown in this table.

than S12TS, indicating that the SrpABC efflux pump contributes to resistance to chloramphenicol, and the resistance is even stronger in S12TS than in the wild-type strain. Since the *srpB* deficient mutant JK1 and the *srpS-srpB* double mutant S12DM showed the same sensitivity to all of the test compounds and antibiotics, it is proposed that SrpS is the specific repressor for the SrpABC efflux pump, and not involved in global regulatory systems.

3.4 Study of the protein-protein interaction between SrpR and SrpS

Since previous evidence indicated that SrpR could probably act as an anti-repressor that derepresses the transcription of the *srpABC* operon by preventing the binding of SrpS to the *srpS-srpA* intergenic region (Zahir 2005), in vivo and in vitro studies were conducted to determine whether these two regulatory proteins interact directly with each other.

3.4.1 In vivo study of the SrpR-SrpS interaction using a bacterial two-hybrid system

A bacterial two-hybrid system based on the interaction-mediated reconstitution of the adenylate cyclase activity in *E. coli* (Karimova et al. 1998) was used to study the interaction between the SrpR and the SrpS proteins. T18 and T25 are the two complementary fragments composing the catalytic domain of adenylate cyclase in

Bordetella pertussis. These two fragments are not active when physically separated, but once they are fused as two interacting polypeptides, the interaction between the two polypeptides results in functional complementation between T18 and T25, and thus cAMP will be synthesized. Since the cAMP/CAP complex turns on the gene expression of the *lac* operon in *E. coli*, the synthesis of cAMP could be detected on LB medium with X-gal.

pKT25 is the plasmid encoding the T25 fragment, while pUT18 and pUT18C are the plasmids encoding the T18 fragment. The only difference between pUT18 and pUT18C is that the MCS is located upstream from the T18 gene in pUT18, but downstream from the T18 gene in pUT18C. The *srpR* and the *srpS* genes were cloned into pKT25, pUT18, and pUT18C, resulting in six recombinant plasmids pKT25-*srpS*, pUT18-*srpR*, pUT18C-*srpR*, pKT25-*srpR*, pUT18-*srpS*, and pUT18C-*srpS*. Four test groups (pKT25-*srpS* + pUT18-*srpR*, pKT25-*srpS* + pUT18C-*srpR*, pKT25-*srpR* + pUT18-*srpS*, and pKT25-*srpR* + pUT18C-*srpS*), one positive control group (pKT25-zip + pUT18C-zip), and one negative control group (pKT25 + pUT18) were set up for the assay. Each pair of plasmids from the test or control groups (e.g. pKT25-*srpS* + pUT18-*srpR*, etc.) were cotransformed into adenylate cyclase deficient (*cya*) *E. coli* strains BTH101 or DHM1. The transformants were plated on LB plates with antibiotics, IPTG and X-gal as described in Chapter 2, and incubated at 30°C, 37°C, or 42°C for up to 72 h. Following the incubation, all colonies on the positive control plates showed dark

blue color. These colonies carried two plasmids pKT25-zip and pUT18C-zip, which had the leucine zipper gene derived from a yeast transcriptional activator fused to the T25 or T18 fragment. On the other hand, all colonies in the test groups and the negative control group showed white color at 30°C and 37°C, but blue color at 42°C. One possible explanation for the blue color at 42°C is that heat shock proteins including molecular chaperones are expressed at this temperature, which can help two poorly interacting proteins to interact and give enzymatic activity to adenylate cyclase. Unfortunately, these results could not be used to prove the interaction between SrpR and SrpS.

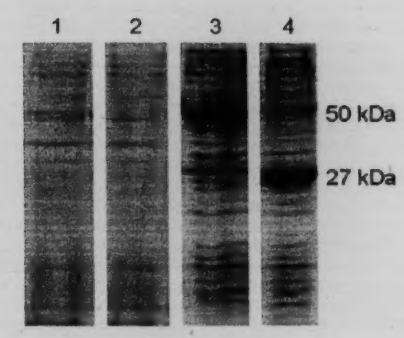
3.4.2 In vitro study of the SrpR-SrpS interaction using the glutathione S-transferase (GST) pull-down assays

To prepare for the GST pull-down assays, *srpR* was cloned into the GST gene fusion vector pGEX-4T-1 (Amersham), and *srpS* was cloned into the 6xHis-tag gene fusion vector pQE31 (Qiagen), according to the procedure described in Chapter 2 (section 2.7.2.1). In addition, *arpR*, the repressor protein gene of the ArpABC efflux pump in *P. putida* S12, was also cloned into pQE31, and used as a negative control for the pull-down assay. *E. coli* BL21 was used to express the GST-tagged proteins, and *E. coli* M15[pREP4] was used to express the 6xHis-tagged proteins. The low-copy plasmid pREP4 (Qiagen), which constitutively expresses the *lac* repressor protein encoded by *lacI*, was introduced into *E. coli* M15 due to the absence of *lac* repressor gene in pQE31.

E. coli cells transformed with the appropriate plasmids were grown, induced, and lysed under the conditions described in Chapter 2 (section 2.7.2.1). In order to ensure the correct expression of the fusion proteins, the induced cell samples containing the overexpressed proteins were analyzed by SDS-PAGE (Fig. 3.11), and the cell lysate samples were analyzed by Western blotting (Fig. 3.12). All of the overexpressed proteins were observed to be of the expected sizes on SDS-PAGE gels (GST-SrpR 50 kDa, GST 27 kDa, His-SrpS 29 kDa, His-ArpR 25 kDa). On the other hand, Western blotting showed that GST-SrpR could be detected by the anti-GST antibody, while His-SrpS and His-ArpR could be detected by the anti-His antibody.

The GST pull-down assay was performed as described in section 2.7.2.4. GST-SrpR was used as the bait protein, and His-SrpS was used as the prey protein. Additionally, three negative control groups were conducted simultaneously: GST-SrpR + His-ArpR, GST + His-SrpS, and pure PBS buffer + His-SrpS. The results of the pull-down assay were analyzed by Western blotting, and Penta-His Antibody was used to detect the 6xHis fusion proteins (Fig. 3.13). A strong band was shown in the test group (lane 1), whereas no band (lane 2) or very faint bands (lanes 3 and 4) were shown in the control groups, indicating that GST-SrpR and His-SrpS specifically interact with each other.

A



B

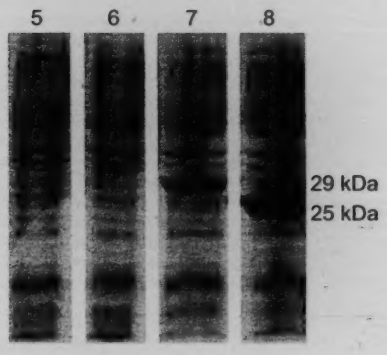


Figure 3.11. SDS-PAGE analysis of the overexpressed GST-tagged and His-tagged fusion proteins. (A) Resuspended cell samples containing the GST-tagged plasmids. Lane 1, uninduced cells containing pGEX-*srpR*; lane 2, uninduced cells containing pGEX-4T-1; lane 3, induced cells containing pGEX-*srpR*; lane 4, induced cells containing pGEX-4T-1. (B) Resuspended cell samples containing the 6xHis-tagged plasmids. Lane 5, uninduced cells containing pQE31-*srpS*; lane 6, uninduced cells containing pQE31-*arpR*; lane 7, induced cells containing pQE31-*srpS*; lane 8, induced cells containing pQE31-*arpR*. Each lane was loaded with 10 µl of uninduced/induced cells resuspended in PBS.

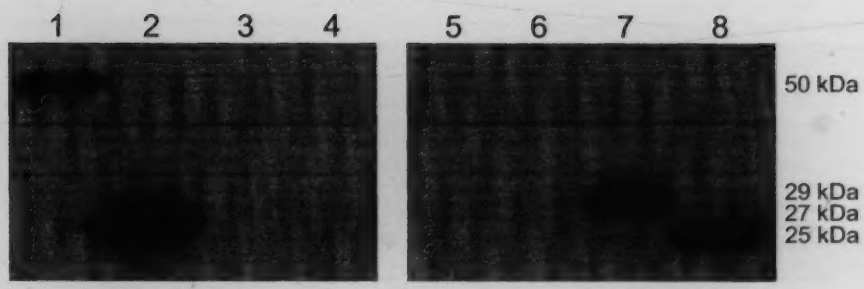


Figure 3.12. Western blot analysis of the overexpressed GST-tagged and His-tagged fusion proteins. Lanes 1 and 5, total protein samples containing GST-SrpR; lanes 2 and 6, total protein samples containing GST; lanes 3 and 7, total protein samples containing His-SrpS; lanes 4 and 8, total protein samples containing His-ArpR. Each lane was loaded with 2.5 μg total protein for SDS-PAGE. After electrophoresis and western transfer, GST and GST-SrpR were detected with Anti-GST-Horseradish Peroxidase Antibody (lanes 1-4); His-SrpS and His-ArpR were detected with Penta-His Antibody and peroxidase-conjugated goat anti-mouse secondary antibody (lanes 5-8).

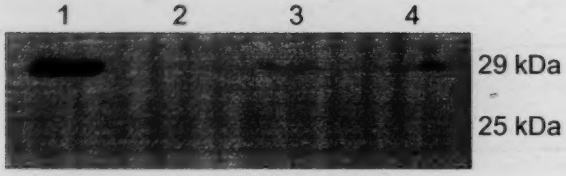


Figure 3.13. Results of the GST pull-down assay shown by Western blotting. The 6xHis fusion proteins were detected by Penta-His Antibody and peroxidase-conjugated goat anti-mouse secondary antibody. Lane 1, GST-SrpR + His-SrpS (test group); lane 2, GST-SrpR + His-ArpR (negative control); lane 3, GST + His-SrpS (negative control); lane 4, PBS buffer + His-SrpS (negative control). The expected size for His-SrpS is 29 kDa, and the expected size for His-ArpR is 25 kDa.

Chapter 4: Discussion

The SrpABC multidrug and organic solvent efflux pump in *P. putida* S12 is a unique RND-type efflux system that has an unusual regulatory system consisting of two local regulatory genes *srpS* and *srpR* located upstream and transcribed divergently away from the structural genes. Although another RND-type efflux system TtgGHI in *P. putida* DOT-T1E is highly homologous to SrpABC, one of its regulatory genes *ttgW* seems to be a pseudogene as it only encodes a protein of 134 amino acids, compared with the 213-amino-acid protein encoded by *srpR* (Ramos 2004; Ramos et al. 2005). As a result, the truncated protein TtgW does not seem to be involved in the regulation of the TtgGHI efflux pump (Rojas et al. 2003), whereas SrpR was shown to derepress the expression of the SrpABC efflux pump (Zahir 2005). The purpose of this study was to further investigate the functions of SrpS and SrpR, and to elucidate how these two regulators act/ interact in the regulation of the SrpABC efflux pump.

Initially, RT-PCR was performed to determine whether *srpS* and *srpR* are polycistronic. Zahir (2005) proposed that these two genes might not be arranged as an operon due to their different transcription levels in the presence of toluene. However, this difference could also be the result of post-transcriptional regulation. On the other hand, Rojas et al. (2003) showed that *ttgV* and *ttgW*, the two regulatory genes of the TtgGHI efflux pump, were transcribed as a single transcriptional unit by performing an RT-PCR analysis. According to the result of our RT-PCR analysis, it was confirmed that *srpS* and *srpR* were indeed polycistronic, since amplification was obtained with the primer pair

across these two genes no matter whether toluene was present or not.

In addition, RT-PCR was also used to determine the transcriptional start sites of *srpS* and *srpA*. The transcriptional start sites of *ttgV* and *ttgG* have been determined previously by Rojas et al. (2003), thus primers upstream or downstream of these sites were designed in the RT-PCR analysis of this study to help determine if these sites were also accurate for the *srpS* and *srpA*. The results confirmed that the transcriptional start sites of *srpS* and *srpA* were the same as the corresponding sites in *ttgV* and *ttgG*, respectively. According to these transcriptional start sites, there is a 42-bp intergenic region between *srpS* and *srpA*, covering both the -10 and the -35 regions of the two genes' promoters. In the future, DNase I or dimethyl sulphate footprint analysis could be performed in this region to identify the exact site that SrpS binds to as it represses the transcription of *srpABC*. Recent studies on TtgABC, another RND-type multidrug efflux pump in *P. putida* DOT-T1E, revealed that the repressor TtgR forms dimers in solution, and two dimers bind to the operator region, which is the intergenic region between *ttgR* and the structural gene *ttgA* (Krell et al. 2007). On the other hand, studies on the TtgGHI efflux pump showed that TtgV exists as tetramers in solution, and binds to the operator in the same oligomeric state. Moreover, TtgV was found to cause a 57° convex bend in its operator DNA, suggesting that TtgV represses the transcription by steric occlusion of the RNA polymerase binding site (Guazzaroni et al. 2007). Similar experiments could be performed in the future to study how SrpS interacts with the *srpS-srpA* intergenic region

and represses the transcription of *srpABC*. More importantly, since SrpR seems to play a functional role in this regulatory system, it would be of great interest to determine how SrpR derepresses *srpABC*'s transcription at the molecular level.

Besides SrpR, substrates of SrpABC such as toluene were proposed to derepress the transcription of the structural genes by binding to the repressor SrpS and altering its conformation to inhibit the binding of SrpS to the operator DNA (Zahir 2005). Consistent with this theory, electrophoretic mobility shift assays showed that 1-hexanol, one of the substrates of TtgGHI, could dissociate the repressor TtgV from its target operator (Guazzaroni et al. 2004). Later, isothermal titration calorimetry (ITC) showed a clear relationship between TtgV's affinity for the efflux pump substrates and their potential to activate the expression of the pump, indicating that the binding constant of the substrates to the pump repressor is a crucial factor for efficient induction of efflux pump gene expression (Guazzaroni et al. 2005). For the SrpABC efflux pump, several antibiotics were found to be substrates, including chloramphenicol, nalidixic acid, and novobiocin, since wild-type S12 had higher MICs to these compounds than did the *srpB*-deficient mutant JK1. This is the first time that not only organic solvents, but also antibiotics have been shown to be substrates of SrpABC. TtgGHI was also observed to play a role in antibiotic extrusion in *P. putida* DOT-T1E, as the mutant lacking both *ttgABC* and *ttgGHI* showed lower MICs to some antibiotics than the mutant lacking only *ttgABC* did (Rojas et al. 2003). Nevertheless, the MICs of the above antibiotics in

wild-type S12 was only twice of that in mutant JK1, indicating that SrpABC does not pump out antibiotics very efficiently. This is probably due to the existence of the other efflux pump ArpABC in S12, which plays a more important role in pumping out antibiotics, but not organic solvents. It is possible that SrpABC is not needed in antibiotic extrusion when ArpABC is working properly. Therefore, to further study SrpABC's function in antibiotic resistance, MIC assays could be repeated in the future with a mutant lacking *arpABC* and a double mutant lacking both *arpABC* and *srpABC*. The result should reveal whether the double mutant is more sensitive to antibiotics than the single mutant. Another experiment that could be done in the future is to determine the binding constant of antibiotics or other compounds to SrpS using ITC. This would help to detect new substrates as well as to confirm previously identified substrates for SrpABC.

The second purpose of the MIC assay in this study was to determine whether SrpS is involved in the regulation of other efflux systems in *P. putida* S12. In order to investigate this, the *srpS-srpB* double mutant strain *P. putida* S12DM was constructed and used in the MIC assays. If SrpS also represses other efflux pumps, the double mutant should have higher MIC values to some of the tested compounds than the single mutant JK1, which also lacks a functional SrpABC efflux pump but has an intact repressor gene *srpS*. However, the result did not show any difference in MICs between S12DM and JK1, indicating that SrpS does not repress the expression of other efflux pumps in *P. putida*

S12, such as ArpABC.

In general, organic solvents with a $\log P_{OW}$ between 1 and 4 are considered toxic to microorganisms and other living cells, as they can be dissolved in the aqueous phase to a relatively high extent and from there partition into the cell membrane (Heipieper et al. 1994; Ramos et al. 2002). However, alcohols with $\log P_{OW}$ values lower than 1 (e.g. *n*-butanol) can penetrate into the bacterial cytoplasm and denature bacterial proteins, so that they are also toxic to living cells at certain concentrations.

Zahir et al. (2006) isolated several Gram positive bacterial strains that are more tolerant to some organic solvents than previously characterized solvent-tolerant Gram negative bacteria. Consistent results were observed when I tested the tolerance of these Gram positive strains to *n*-butanol (see Chapter 6). Four Gram positive strains were used in this test, including *Staphylococcus* sp. strain ZZ1, *Bacillus cereus* strain ZZ2, *B. cereus* strain ZZ3, and *B. cereus* strain ZZ4. All the four strains were able to grow rapidly in LB broth with 1% (v/v) *n*-butanol, while wild-type *P. putida* S12 could not survive the same condition at all. From this result, it is obvious that both the two characterized efflux pumps in *P. putida* S12 failed to help it survive this *n*-butanol shock. On the other hand, the strong solvent tolerance demonstrated in these Gram positive strains still remains uncharacterized. Efflux pumps could be one of the possible mechanisms, while the extracellular capsule observed by electron microscope in strain ZZ1 could also contribute to this trait. More investigation is needed in the future to

further characterize these organic solvent-tolerant Gram positive bacteria.

According to Wery et al. (2001), 93% of the *P. putida* S12 cells that survived a 1% (v/v) toluene shock had their *srpS* gene interrupted by the IS*S12* insertion sequence. However, totally different results were obtained in our study when I repeated these experiments in a similar way. No insertion sequences were detected when the cells were treated with 1% (v/v) toluene shocks. Further studies showed that about 1/3 of the cells that survived a 20% (v/v) toluene shock carried a new insertion sequence designated as IS*PpuS12* within *srpS*. This newly-identified insertion sequence was found to have three copies in wild-type *P. putida* S12, while the one located within *srpS* was an extra copy. Mutant S12TS, carrying an extra copy of IS*PpuS12* within *srpS*, has a 17000-fold increased survival frequency to toluene shock in comparison with wild-type S12. The reason for this result is probably that the SrpABC efflux pump is expressed constitutively at a high level in mutant S12TS due to the lack of the repressor gene *srpS*, so that the mutant is more prepared for toluene shock compared with the wild-type strain. Later, it was found that the increased survival frequency in S12TS dropped down to the wild-type level once it was complemented with *srpS*, while the complementation by *srpR* alone did not affect the survival frequency. This result is consistent with the hypothesis that only SrpS, but not SrpR, is the repressor of SrpABC, while the results reported by Wery et al. (2001) are incorrect, as they claim that the complementation by both *srpR* and *srpS* is necessary to make the IS mutant as sensitive to toluene as the wild-type strain. In

addition, our study showed that 2/3 of the cells that survived toluene shock did not have an *srpS* gene interrupted by insertion sequences. This finding does not support Wery et al.'s (2001) conclusion that the solvent shock tolerant nature of *P. putida* S12 lies in the absolute presence of insertion sequences in the *srpS* gene in a small minority of its population. Based on our results, I conclude that the presence of insertion sequences in *srpS* is a random event, and that the solvent-tolerant nature of *P. putida* S12 may alternatively rely upon a rapid response by the SrpS-SrpR regulatory system.

Zahir (2005) studied the *srpA* promoter activity in SrpS-deficient and SrpR-deficient backgrounds by constructing *srpA-lacZ* reporter gene fusion strains and performing β-galactosidase assays (Table 1.2). It was shown that expression of the *srpA* promoter increased 5.5-fold in the SrpS-deficient mutant, but decreased fivefold in the SrpR-deficient mutant, indicating that SrpS represses whereas SrpR derepresses the expression of the *srpABC* operon. In addition, gel mobility shift assays showed that SrpS binds to the *srpS-srpA* intergenic region and represses the transcription of *srpA*. On the other hand, SrpR does not bind to the same DNA region, but it inhibits the binding of SrpS to the operator, suggesting that the derepressing effect of SrpR is achieved by the direct interaction between SrpR and SrpS. To further investigate this, I conducted in vivo and in vitro studies to determine the protein-protein interaction between these two regulators. Initially, a bacterial two-hybrid system based on the reconstitution of *Bordetella pertussis* adenylate cyclase was used as an in vivo method. The process and

result of this experiment were described in Chapter 3. Unfortunately, this system failed to detect the interaction between the two regulatory proteins. One possible reason for this failure to detect interaction is that the structure of SrpS and SrpR might not remain in their natural state once they are fused to the two complementary fragments of the adenylate cyclase catalytic domain. Since proper folding of each of the interacting partners is very important to facilitate protein-protein interaction, this two-hybrid system might not be suitable for detecting the interaction between SrpS and SrpR. Another reason for this failure could be that the protein interaction is not strong enough to be detected by this two-hybrid system, as the interaction between SrpS and SrpR might be relatively weak and in a dynamic equilibrium which facilitates a rapid response to the influx of organic solvents or other toxic compounds.

Subsequently, GST pull-down assays were used as an in vitro method to determine the protein-protein interaction between SrpR and SrpS. The results showed that the bait protein GST-SrpR specifically binds the prey protein His-SrpS but not the negative control His-ArpR, the repressor protein for the ArpABC efflux pump. Therefore, this in vitro evidence supports the hypothesis that SrpR directly binds to SrpS and acts as an anti-repressor in the transcriptional regulatory system of the SrpABC efflux pump.

As described in Chaper 1, SrpR was predicted to belong to the TetR family of transcriptional regulators. All members of this family whose function is known are transcriptional repressors, and they have a high degree of amino acid similarity at the

HTH DNA-binding domain, which is used as the signature domain to define this regulator family (Ramos et al. 2005). Although SrpR also contains this HTH domain in its amino acid sequence, the results from previous experiments (Zahir 2005) and this study showed that it is neither a DNA-binding protein nor a transcriptional repressor. Thus, whether SrpR functionally belongs to the TetR family remains in question. Further studies should be carried out to confirm the protein-protein interaction between SrpR and SrpS. One of the feasible approaches would be to develop antibodies against SrpR or SrpS, and perform co-immunoprecipitation with whole cell lysates. On the other hand, it would be interesting to investigate whether SrpR or SrpS can bind to other DNA regions around the *P. putida* chromosome and repress/ activate the transcription of other genes. The chromatin immunoprecipitation (ChIP)-on-chip technique may be helpful to identify the DNA binding sites of these regulators, if there are any, among the genome of *P. putida* S12.

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Chapter 6: Appendix

6. Appendix: Survival test of several Gram positive and Gram negative bacterial strains in *n*-butanol

6.1 Materials and methods

6.1.1 Bacterial strains

The bacterial strains used in this experiment are listed in Table 6.1.

6.1.2 Culture conditions

A single colony of each of the bacterial strains was used to inoculate 5 ml LB broth with 1% (v/v) butanol and the culture was grown at 30ºC or 37 ºC with shaking at 200 rpm for up to 48 h.

6.2 Results

The results of this experiment are listed in Table 6.2. All the four tested Gram positive strains were able to grow rapidly (within 24 h) at both 30ºC and 37ºC in the presence of 1% (v/v) *n*-butanol, whereas none of the tested Gram negative strains except *E. coli* DH5α could grow in the same condition after 48 h incubation.

Table 6.1. Bacterial strains used in the *n*-butanol survival experiment

Strain	Relevant characteristics	Reference / source
Staphylococcus sp. strain ZZ1	Organic solvent-tolerant Gram positive bacterial strain.	(Zahir et al. 2006)
Bacillus cereus strain ZZ2	Organic solvent-tolerant Gram positive bacterial strain.	(Zahir et al. 2006)
B. cereus strain ZZ3	Organic solvent-tolerant Gram positive bacterial strain.	(Zahir et al. 2006)
B. cereus strain ZZ4	Organic solvent-tolerant Gram positive bacterial strain.	(Zahir et al. 2006)
E. coli DH5α	F^- φ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen
P. putida S12	Organic solvent-tolerant Gram negative bacterial strain. Wild-type, *srpABC*$^+$, *srpR*$^+$, *srpS*$^+$	(Hartmans et al. 1990)
P. putida S12TS	Organic solvent-tolerant Gram negative bacterial strain. *srpS*::IS*PpuS12*	This study
P. putida JK1	Organic solvent-sensitive Gram negative bacterial strain. *srpB*::Tn*Mod*-KmO	(Kieboom et al. 1998a)
P. putida PPO200	Organic solvent-sensitive Gram negative bacterial strain.	(Zylstra et al. 1989)

Table 6.2. Growth of several Gram positive and Gram negative bacterial strains in LB broth with 1% (v/v) *n*-butanol

Strain	Growth in LB with 1% (v/v) *n*-butanol	
	30°C	37°C
Gram +ve:		
Staphylococcus sp. strain ZZ1	++	++
B. cereus strain ZZ2	++	++
B. cereus strain ZZ3	++	++
B. cereus strain ZZ4	++	++
Gram –ve:		
E. coli DH5α	++	+
P. putida S12	–	–
P. putida S12TS	–	–
P. putida JK1	–	–
P. putida PPO200	–	–

++ = Growth within 24 h; + = growth within 48 h; – = no growth.